REMARKS

After entry of this amendment, claims 1 and 3-34 are pending, of which claims 11-20 and 27-31 are withdrawn. Claim 1 has been amended without prejudice or disclaimer. Support is found *inter alia* in the original claim. No new matter has been added. Applicants respectfully request entry of the above claim amendment as it is believed to put the claims in condition for allowance or, alternatively, in better form for consideration on appeal. Thus, entry under 37 CFR §1.116 is correct.

Claim Objection

The Examiner objects to claims 32 and 33 for being drawn to non-elected inventions. Applicants respectfully disagree. Claims 32 and 33 are dependent from claim 1, directly or indirectly, and as such are consistent with elected Group I drawn to a process for preparing transformed plant cells or organisms. Applicants realize that claims 32 and 33 contain non-elected species and only the species listed under part a) corresponds to the elected species. Pursuant to 37 CFR § 1.141, if a claim generic to all the claimed species has been allowed and all the claims to species depend from an allowable generic claim, claims to additional species which recite all the limitations of the generic claim may be rejoined. Accordingly, Applicants respectfully request rejoinder of the remaining species if claim 1 is found allowable.

Claim Rejections – 35 USC § 112

Indefiniteness rejection

Claims 1, 3-10, 21-26, and 32-34 are rejected under 35 U.S.C. § 112, second paragraph, for indefiniteness. Claim 1 has been amended without prejudice or disclaimer as suggested by the Examiner. No new matter has been added. In light of the amendment, the rejection is believed to be rendered moot. Reconsideration and withdrawal of the rejection is respectfully requested.

Written description rejection

Claims 1, 3-4, 7-10, 25-26, and 32-34 stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the written description requirement. The Examiner asserts that, although the specification provides 17 candidate proteins, neither the specification, nor the prior art, provides any guidance as to whether those claimed "toxic

compounds" are actually toxic to plant cells under the condition used for transformation in the presence of corresponding marker proteins. The Examiner further asserts that the specification does not provide guidance as to what concentration of compounds should be used for selection in plant transformation. Applicants respectfully disagree.

As disclosed in the specification at page 12, lines 31-33, the marker proteins are preferably the "negative selection markers" as used in the art. Examples of marker proteins and their mode of actions are detailed at pages 12-20. Their use as "negative selection markers" is well known in the art. For example, the use of alcohol dehydrogenase (part (n) of claim 32) as negative selection marker protein in plants can be traced back to 1975 (Schwartz et al., Genetics, 1976, 83: 63-65). The use of cytosine deaminase (part (a)) and cytochrome P-450 enzyme (part (b)) as negative selection markers in transgenic barley is also disclosed in Koprek et al. (Plant J., 1999, 19: 719-726). Similarly, Upadhyaya et al. (Plant Mol. Biol. Reporter, 2000, 18: 227-233) discloses the use of an indoleacetic acid hydrolase (*tms2* gene product, part (c)) as negative selection marker in rice. Likewise, Naested et al. (Plant J., 1999, 18: 571-576) discloses the use of a haloalkane dehalogenase (part (d)) as a negative selection marker in Arabidopsis. The use of a thymidine kinase (part (e)) as a negative selection marker in Arabidopsis is also shown in Czakó et al. (Plant Physiol., 1994, 104: 1067-1071). A copy of each aforementioned article is attached for the Examiner's easy reference.

Additionally, the use of indoleacetamide hydrolase (part (i)) and indoleacetamide hydrolase (part (j)), as well as the above-mentioned marker proteins, as negative selection markers is disclosed in US 7,288,694, Col. 2, lines 29-67. Similarly, the use of xanthine/guanine phosphoribosyl transferase (part (f)), phosphonate monoester hydrolase (part (h)), methoxinine dehydrogenase/rhizobitoxin synthase (part (l)), purine nucleoside phosphorylase (part (g)), and adenine phosphoribosyl transferase (part (k)) as negative selection markers is also disclosed in US 6,576,443, US 5,254,801, US 7,371,935, US 5,464,764, and US 5,583,278, respectively. Additionally, the effect of methylthioribose kinase and (trifluoromethyl)thioribose (part (m)) in cell growth and the potential use as negative selection marker is also discussed in Sekowska et al. (BMC Microbiology, 2002, 2:8, copy attached).

Accordingly, it is respectfully submitted that the specification, together with the knowledge of the art, correlate the marker proteins with the function of being able to be used for

selection in plant transformation. Reconsideration and withdrawal of the rejection is respectfully requested.

Moreover, Applicants wish to note that the claimed subject matter relates to a process for preparing transformed plant cells or organisms using dsRNA. As set forth in *Vas-Cath Inc. v. Mahurkar*, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991), the test for determining compliance with the written description requirement is whether the disclosure of the application as originally filed reasonably conveys to one skilled in the art that the inventor had possession of the claimed subject matter at the time of filing. Thus, the question here is whether Applicant possessed the claimed **process**.

According to the "Guidelines for the Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1, 'Written Description' Requirement," at page A-6, 3rd column of the "Written Description Training Materials" (the "Guidelines," revision of March 25, 2008), possession of an invention can be shown "in a variety of ways, **including description of an actual reduction to practice**" (emphasis added). The present application describes an actual reduction to practice of the claimed process by working examples. As the Examiner noted in the Office Action at page 5 (under 'Enablement" section), the specification describes and is enabling for the CodA marker protein and the dsRNA of CodA. Thus, possession of the claimed process is shown by its reduction to practice, and the rejection should be withdrawn.

Enablement rejection

Claims 1, 3-4, 7-10, 25-26, and 32-34 stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly lacking an enabling disclosure. The Examiner asserts that the specification is not enabling for any marker proteins capable of causing directly or indirectly a toxic effect for plant cells by any means. Applicants respectfully traverse.

As discussed above, the claimed subject matter relates to a **process** and the individual marker proteins or the coding genes are not being claimed. Rather, the claimed subject matter concerns an improved selection method for preparing transformed plant cells. Thus, the "nature of the invention" *Wands* factor is the process, not the marker proteins used in the process.

As to the scope of the claims, it is correct that the broadest claims encompass any marker proteins capable of causing a toxic effect in plant cells. However, the need for routine

experimentation to identify suitable concentration ranges of the substance X and conditions for expressing the marker proteins useful for selecting transformed plant cells in the claimed process does not defeat enablement. *In re Wands*, 8 USPQ 2d, 1400, 1404 (Fed. Cir. 1988) ("Enablement is not precluded by the necessity for some experimentation such as routine screening."); see also *Ex parte Kubin*, 83 USPQ2d 1410, 1416 (B.P.A.I. 2007) (although practicing the full scope of the claims might have required extensive experimentation, the experimental techniques were well-known in the art, so the experimentation would have been routine and thus, not undue). Thus, the "amount of experimentation" *Wands* factor favors enablement.

Furthermore, the specification, by way of Examples, describes how to make and use the claimed process, illustrating an actual reduction to practice. Thus, the "presence of absence of working examples" *Wands* factor clearly supports enablement.

Moreover, it is noted that the level of skill is high, which further supports a finding of enablement.

With regard to the "amount of guidance" *Wands* factor, the Examiner asserts that the specification fails to provide guidance required to practice the claimed process with all the marker proteins, except for the one specifically described in the specification. Particularly, the Examiner alleges that the specification does not provide information such as gene sequence encoding marker proteins and concentration of the substance X to be used for selection in the claimed process. Applicant respectfully disagrees with the Examiner's characterization of the claimed subject matter.

As discussed above, negative selection marker proteins are known in the art. For example, the references discussed above in the "written description" section provide detailed information relating to the gene sequence encoding marker proteins and concentration of the substance X to be used in the respective negative selection. See e.g., Koprek et al. As also noted, the operability of the process is shown by reduction to practice, and Applicants submit that no basis exists to question the operability of the process using other marker proteins. Furthermore, screening for reduced marker protein expression due to the effect of the dsRNA expression is simply routine experimentation, and not undue. Because the claimed subject matter can be carried out by means of standard procedures which are well known to one skilled

in the art, it is submitted that the specification sufficiently enables a person of ordinary skilled in the art to make and/or use the present invention without undue experimental burden.

In view of the detailed description, guidance, working examples, and high level of skill, the specification enables the claimed method. On these facts, an analysis under *In re Wands* supports enablement. *In re Wands*, 858 F.2d at 737 (routine screening of hybridomas was not "undue experimentation;" the involved experimentation can be considerable, so long as "routine"). Note that the test for whether experimentation is "undue" is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the invention claimed. *Ex parte Jackson*, 217 USPQ 804, 807 (1982). For at least these reasons, undue experimentation is not required to make and use the claimed method. Reconsideration and withdrawal of the rejection is respectfully requested.

Claim Rejections – 35 USC § 103

Claims 1, 3-10, 21-26 and 32-34 are further rejected as unpatentable over Maliga et al. (WO 01/21768, hereinafter "Maliga") in view of Smith et al. (Nature, 2000, 407: 319-320, hereinafter "Smith"), taken with admissions in the specification. Applicants respectfully disagree and traverse the rejection for the reasons described in the response dated November 15, 2007 and for the following additional reasons.

Applicants initially note that the claimed subject matter is directed to a reversed negative selection method rather than a "double selection" method as alleged by the Examiner (see Office Action at page 8, last paragraph). The selection method, according to the present application, consists of (1) providing plants which express negative marker gene, (2) transforming said plants with a construct containing a gene of interest and a dsRNA or an expression cassette expressing a dsRNA directing to the marker protein gene (which interfere with expression of the marker protein), (3) adding the pre-toxin (substance "X"), and (4) selecting surviving plants based on the ability to grow in the presence of an otherwise toxic concentration of substance "X." This method therefore allows the use of any known negative selection markers to be used as "positive selection markers" in a selection method to select transgenic plants and it is not described in the state of the art until the present application.

For instance, although using codA as negative selection marker is known in the art and Maliga teaches cells lacking codA could be readily identified by 5-FC resistance, the site-specific recombination system taught in Maliga is aimed to **remove** the selection marker gene from the plastid genome once the transformed plant progeny is successfully isolated (see page 3, lines 6-9). Maliga does not teach or suggest that the effect of the selection marker gene could be reduced by using dsRNA of the marker protein encoding gene **without removing** the gene out of the genome. Nor does Maliga teach or suggest the use of codA for the "positive" selection method as claimed.

Smith does not remedy such a deficiency. Smith teaches a DNA construct that produces a hairpin loop type dsRNA with functional (*i.e.* spliceable) intron as a spacer and its usefulness to induce post-transcriptional gene silencing (PTGS). Smith does not teach or suggest that PTGS may be used or useful in a transgenic plant selection system. It follows that neither Maliga nor Smith, alone or combined, discloses or suggests transforming a plant expressing a negative marker gene with a construct containing a gene of interest and a marker protein dsRNA-expression cassette as required by the present invention. Accordingly, neither document nor their combined teaching suggests the reversed negative selection as disclosed in the present application that allows the use of any known negative selection markers to be used as "positive selection markers."

The Examiner further alleges that it would have been obvious to try using the hairpin structure taught in Smith to reduce the expression of codA gene, and the resulting plant would have had reduced expression of codA gene as expected and be resistant to 5-FC. The motivation to do so, according to the Examiner, comes from the general knowledge of the art that it would be more effective to have double selection to select true transformants. Applicant respectfully disagrees with the Examiner's characterization and conclusion.

A claim would have been obvious if all the claimed elements were known in the prior art and one skilled in the art could have combined the elements as claimed by known methods with no change in their respective functions, and the combination would have yielded nothing more than predictable results to one of ordinary skill in the art at the time of the invention. However, as the court in *KSR* pointed out, "it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the

claimed new invention does." *KSR International Co. v. Teleflex Inc.*, 82 USPQ2d 1385, 1396 (2007). Thus, "rejections on obviousness cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *Id.*, quoting *In re Kahn*, 441 F.3d 977, 988, (Fed. Cir. 2006). As discussed above, the claimed subject matter is directed to specific selection steps using, e.g. codA. The art does not teach, suggest, or motivate the specific series of steps which constitute an unobvious way of using the marker protein genes. Accordingly, the combination of Maliga and Smith, even taken together with the knowledge of the art, does not support a finding of obviousness.

For at least the above reasons, the subject matter of the pending claims would not have been obvious. Reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION

In view of the amendments and the reasons presented above, reconsideration of the rejections and allowance of the claims is respectfully requested. If any outstanding issues remain, the Examiner is invited to telephone the undersigned at the number given below.

Applicants wish to draw the Examiner's attention that the European counterpart of the present application, EP1 527 183, has been recently granted by the European Patent Office.

Accompanying this response is a petition for a two-month extension of time to and including July 22, 2008 to respond to the Office Action mailed February 22, 2008 with the required fee payment. No further fee is believed due. However, if any additional fee is due, the Director is hereby authorized to charge our Deposit Account No. 03-2775, under Order No. 12810-00057-US from which the undersigned is authorized to draw.

Respectfully submitted,

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Attachments:

1. Schwartz et al., A pollen selection system for alcohol-dehydrogenase-negative mutants in plants. Genetics, 1976, 83: 63-65.
2. Koprek et al., negative selection systems for transgenic barley (*Hordeum vulgare* L.): comparison of bacterial codA- and cytochrome P450 gene-mediated selection. Plant J., 1999, 19: 719-726.
3. Upadhyaya et al., The *tms2* gene as a negative selection marker in rice. Plant Mol. Biol. Reporter, 2000, 18: 227-233.
4. Naested et al., A bacterial haloalkane dehydrogenase gene as a negative selection marker in *Arabidopsis*. Plant J., 1999, 18: 571-576.
5. Czakó et al., The herpes simplex virus thymidine kinase gene as a conditional negative-selectioin marker gene in *Arabidopsis thaliana*. Plant Physiol., 1994, 104: 1067-1071.
6. Sekowska et al., The methionine salvage pathway in *Bacillus subtilis*. BMC Microbiology, 2002, 2: 8.

A POLLEN SELECTION SYSTEM FOR ALCOHOL-DEHYDROGENASE-NEGATIVE MUTANTS IN PLANTS

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ABSTRACT

Exposure of freshly shed maize pollen to allyl alcohol vapors allows selective fertilization by mutant alcohol-dehydrogenase-negative grains. Wild-type pollen grains are killed by the enzymatic conversion of allyl alcohol to the highly toxic acrylaldehyde.

MUTANT selection is a powerful tool in genetic analysis. The great strides made in the field of microbial genetics would not have been possible without the procedures by which specific, infrequently occurring mutant forms could be preferentially selected out of a large population of cells. The purpose of this communication is to describe a pollen selection system for alcohol dehydrogenase (ADH) mutants in plants. Pollen grains with the wild-type alleles are killed and only grains which are ADH negative are functional.

Selection procedures for ADH-negative mutants have been previously described by MEGNET (1967) with Saccharomyces, and by SOFER and HATKOFF (1972) with Drosophila. MEGNET grew yeast on medium containing allyl alcohol. ADH converts allyl alcohol to the highly toxic acrylaldehyde. Wild-type cells which contain active enzyme are killed by the acrylaldehyde, while mutant cells which lack ADH activity and cannot carry out the conversion are not affected. SOFER selected ADH-negative Drosophila by treating the flies with 1-pentene-3-ol which is oxidized by ADH into a highly toxic ketone.

The process we developed with maize involves treatment of mature pollen grains with allyl alcohol. Alcohol dehydrogenase is synthesized and found in the mature pollen grains. The enzyme is not needed for pollen germination and growth (SCHWARTZ 1969). Enormous numbers of pollen grains are produced by the maize plant, a single tassel producing more than 18 million. Large numbers of grains can be applied to the silk for fertilization. The silk is itself stigmatic and is covered with numerous stigmatic hairs. Many pollen grains can germinate on the same silk but usually only one functions in fertilization. Thus, large numbers of pollen grains can be treated with allyl alcohol and placed on the silks. The selection technique is powerful in that regardless of the number of grains treated and applied to the silk only the ADH-negative grains survive the treatment and will function in fertilization. All kernels formed on the ears receive an ADH-negative allele from the male parent.

For the treatment of pollen with allyl alcohol we constructed an airtight plexiglass box 12″ × 12″ × 12″ with a shallow drawer 24″ × 6″ which can be

moved in and out of the box from opposite sides. A given amount of allyl alcohol pipetted onto a glass plate is placed into the drawer, moved into the box and allowed to vaporize. A sheet of paper cut to size is placed on the exposed section of the drawer and freshly shed pollen spread in a thin layer. After the allyl alcohol has vaporized the pollen is moved into the box and allowed to remain in the presence of the allyl alcohol for a prescribed period of time. Treatment consists of five-minute exposure to the vapors from 0.03 ml of allyl alcohol. Following this treatment pollen grains with ADH fail to germinate and can not achieve fertilization. However, pollen grains collected from mutant ADH-negative plants readily survived the treatment. Germination is not affected and the fertilized ears show a full seed set. The most satisfactory treatment was determined by testing treated wild-type and mutant pollen grains for germination on supplemented agar (COOK and WALDEN 1965). To insure against contamination by untreated pollen, plants to be used as females are grown in an isolation field and detasseled daily. This technique makes it relatively simple to obtain new null-type mutants at the *Adh* locus. One of us (D.S.) has been using this method to select for a rare ADH$^-$ recombinant resulting from an intragenic crossover between two *Adh* alleles each of which forms active enzyme. A total of 34 seeds were found on 276 ears pollinated with grains produced by plants heterozygous for the two active alleles. Since kernels are sacrificed in the test for *Adh* genotype, plants are first being grown from these kernels and the tests will be performed on their progeny. One of us (J.O.) has been using this method to select cases where the *Ds* element (MCCLINTOCK 1950) has been transposed to the *Adh* locus, for study of the mechanism of gene suppression by this controlling element. Pollen was collected from plants which carried one dose of activator *Ac* and *Ds* present at either the *Bz* locus (*bz-m2*) on chromosome 9 (MCCLINTOCK 1951) or at the *Bz2* locus (*bz2-m*) on chromosome 1 (NUFFER 1955). The treated pollen was applied to silks of plants with specific *Adh* genotypes. *Ds* transposition to, and suppression of the *Adh* gene in the pollen will allow the grains to survive the allyl alcohol treatment and function in fertilization. In the cross with treated *bz-m2* pollen 178 seeds were produced on 265 ears, and in the *bz2-m* crosses 146 seeds were produced on 160 ears. Twelve kernels from the *bz-m2* crosses were sacrificed for electrophoretic analysis of the *Adh* isozymes in the embryo. The female parent was of the genotype *AdhS/AdhS* and the pollen carried the *AdhF* allele (SCHWARTZ and ENDO 1966). Eleven kernels showed only the SS ADH band, indicating complete suppression of the *AdhF* allele introduced through the pollen. The twelfth kernel showed all three isozyme bands, FF, FS, and SS but the ratio was strikingly skewed toward SS. This is the result expected from subsequent derepression of the *AdhF* gene by an *Ac*-mediated transposition of *Ds* from the *Adh* locus.

An early transposition of *Ds* to the *Adh* locus in the sporophyte could give rise to a sector of tassel with many anthers having ADH-negative pollen grains. Thus, the 324 kernels obtained in these crosses did not result from that many transpositions of *Ds* to the *Adh* locus, and many could have resulted from the same early transposition event.

Supported by research grant NSF GB 35462X (to D.S.) and training grant PHS TO1 GM 82-15 (to J.O.). The assistance of LYNN PETERSON is gratefully acknowledged.

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Corresponding editor: J. LAUGHNAN

The Plant Journal (1999) **19**(6), 719–726

TECHNICAL ADVANCE

Negative selection systems for transgenic barley (*Hordeum vulgare* L.): comparison of bacterial *cod*A- and cytochrome *P450* gene-mediated selection

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Summary

Efficient negative selection systems are increasingly needed for numerous applications in plant biology. In recent years, various counter-selectable genes have been tested in six dicotyledonous species, whereas there are no data available for the use of negative selection markers in monocotyledonous species. In this study, we compared the applicability and reliability of two different conditional negative selection systems in transgenic barley. The bacterial *cod*A gene encoding cytosine deaminase, which converts the non-toxic 5-fluorocytosine (5-FC) into the toxic 5-fluorouracil (5-FU), was used for *in vitro* selection of germinating seedlings. Development of *cod*A-expressing seedlings was strongly inhibited by germinating the seeds in the presence of 5-FC. For selecting plants in the greenhouse, a bacterial cytochrome *P450* mono-oxygenase gene, the product of which catalyses the dealkylation of a sulfonylurea compound, R7402, into its cytotoxic metabolite, was used. T_1 plants expressing the selectable marker gene showed striking morphological differences from the non-transgenic plants. In experiments with both negative selectable markers, the presence or absence of the transgene, as predicted from the physiological appearance of the plants under selection, was confirmed by PCR analysis. We demonstrate that both marker genes provide tight negative selection; however, the use of the *P450* gene is more amenable to large-scale screening under greenhouse or field conditions.

Received 30 April 1999; revised 9 July 1999; accepted 19 July 1999.
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Introduction

The expression of negative selection genes causes either immediate or conditional cell death under specific developmental or environmental conditions and therefore allows for the selection of cells lacking these marker genes. These genes have been used successfully to study processes of development and differentiation (Mariani *et al.*, 1992), to stabilize transposable elements (Renckens *et al.*, 1992), to select homologous recombination events in gene targeting experiments (Thykjær *et al.*, 1997) and to develop new strategies for engineering disease-resistant plants (Strittmatter *et al.*, 1995). The use of negative selection genes requires a level of marker gene expression sufficient to cause either death of the expressing cells or significant morphological change in the whole organism. Such genes can be non-conditional or conditional. Various non-conditional marker genes have been tested in higher plants, for example the *Corynebacterium* diphtheria toxin A-chain gene in tobacco (Koltunow *et al.*, 1990) and *Arabidopsis* (Czakó *et al.*, 1992), the *Pseudomonas* endotoxin A gene in *Brassica napus* (Koning *et al.*, 1992) and the *anti-nptII* gene in tobacco (Xiang and Guerra, 1993). The expression of these genes results in immediate lethality in the plant after transformation except where the genes are driven by tissue-specific or inducible promoters. Enzymes encoded in conditional marker genes exhibit an alternative control mechanism for counter-selection schemes by metabolizing an externally provided substrate into its phytotoxic derivative. The utility of several conditional markers has been confirmed in *in vitro* studies; these include *tms2* in tobacco (Depicker *et al.*, 1988) and in *Arabidopsis* (Karlin-Neuman *et al.*, 1991), *aux2* in cabbage (Beclin *et al.*, 1993), the HSV-tk gene in tobacco (Czakó and Márton, 1994) and a cytochrome *P450* gene in tobacco (O'Keefe *et al.*, 1994). Among these markers, the cytosine deaminase gene from *E. coli*, *cod*A, which converts the non-toxic 5-fluorocytosine (5-FC) into the toxic 5-fluorouracil (5-FU), has proven to be efficient and reliable for the counter-selection of transformed protoplasts, callus and seedlings of *Arabidopsis thaliana* (Kobayashi *et al.*, 1995; Perera *et al.*, 1993), *Lotus japonicus* (Stougaard, 1993), *Nicotiana sylvestris* (Stougaard, 1993) and *Nicotiana tabacum* (Schlaman and Hooykaas, 1997). The lack of endogenous cytosine deaminase activity in many

plant species, e.g. *Arabidopsis*, soybean, sugarbeet, barley and pea, indicates the potential wide applicability of *cod*A as a negative selectable marker (Stougaard, 1993).

The bacterial *cod*A gene encoding cytosine deaminase could only be used in the aforementioned studies for *in vitro* selection of germinating seedlings. For selecting plants in the greenhouse, a bacterial cytochrome *P450* mono-oxygenase gene, the product of which catalyses the dealkylation of a sulfonylurea compound into its cytotoxic metabolite, can be used. *In vitro* studies showed that the cytochrome *P450* mono-oxygenase gene from *Streptomyces griseolus* is functional in the presence of specific chloroplast oxidoreductases belonging to higher plants (O'Keefe *et al.*, 1991, 1993). Targeting of the *P450* monooxygenase to the chloroplast in transgenic tobacco (O'Keefe *et al.*, 1994) resulted in an efficient dealkylation of a sulphonylurea-based selective agent, R7402, converting it into its toxic derivative. To date, *P450* mono-oxygenase is the only negative selectable marker gene that has been used in spray tests on fully developed transgenic plants (O'Keefe *et al.*, 1994). Progress in monocot transformation during the past few years has provided the means to apply new molecular strategies to important cereals, e.g. rice, wheat, maize and barley.

Despite the utility of these new methods for studying and modifying cereals and the potential for the broad applicability of negative selection systems in these crop plants, no reports on the use of available negative marker genes has been published in monocots. In this report, we describe the successful application of two negative selection systems in transgenic barley. We analysed T_1 and T_2 plants of several independently transformed barley lines with regard to the effectiveness and reliability of the bacterial *cod*A and *P450* genes in negative selection protocols.

Results

Sensitivity of transgenic and non-transgenic embryos to 5-FC

Studies were conducted to determine the 5-FC concentration needed to distinguish between *codA*-positive and *codA*-negative embryos; embryos of a defined size (2.0–2.5 mm) were used in all germination tests. The germination of tissue culture-derived non-transgenic barley embryos was not significantly inhibited by concentrations of up to 3 mg ml^{-1} of 5-FC (Table 1); higher concentrations of 5-FC reduced germination frequency and resulted in abnormal embryo development in some cases (data not shown). At a concentration of 1 mg ml^{-1} 5-FC, germination and growth of transgenic embryos was visually inhibited.

With respect to embryos from transgenic plants, inhibition of germination and growth could be observed at 1 mg ml^{-1} 5-FC. About half (52%) of the embryos germinated on 1 mg ml^{-1} 5-FC and among these were several embryos with abnormal development. At a concentration of 2 mg ml^{-1}, most embryos showed elongation of the embryo-axis 3 days after transfer to selective medium. In about 70% of the embryos, development halted at day 5 and germination did not occur until day 10. Twenty-nine per cent of the embryos germinated at day 7 and exhibited normal shoot development. The observed sensitive to tolerant ratio at 2 mg ml^{-1} (142:58; Table 1) is consistent with the expected ratio of 3:1 for a single copy of a dominant resistance gene in hemizygous plants. The use of 3 mg ml^{-1} 5-FC resulted in a similar ratio (142 sensitive to 40 tolerant; Table 1). Higher concentrations of 5-FC reduced the germination frequency of non-transgenic embryos markedly. A level of 2 mg ml^{-1} was chosen as the concentration of choice for further experiments. In general, seedlings sensitive to 5-FC did not recover after 7 days of treatment with 5-FC when transferred to medium without 5-FC but did recover prior to 7 days (data not shown). At a concentration of 20 μg ml^{-1}, 5-FU, the toxic metabolite produced by the action of the *cod*A gene product on 5-FC, halted embryo development at a very early stage in transgenic as well as in non-transgenic embryos. Figure 1 shows the effects of various concentrations of 5-FC and 5-FU on transgenic as well as non-transgenic embryos. It was observed that embryo size affected the reliability of the selection scheme; embryo sizes between 2 and 2.5 mm worked most efficiently and were used to generate the data in Table 1.

Sensitivity of transgenic and non-transgenic plants to R7402

Non-transgenic plants were only slightly affected when sprayed with concentrations of R7402 up to 100 μg m^{-2}. A concentration of 250 μg m^{-2} reduced average shoot length of non-transgenic plants by about 20%; higher concentrations resulted in individual plants with strongly reduced shoot length, similar to that of transgenic plants expressing the transgene (Table 2). Development of transgenic plants was inhibited after treatment with 50–250 μg m^{-2}. An increase in R7402 concentration above 250 μg m^{-2} caused little additional damage.

Use of concentrations of R7402 between 50 and 250 μg m^{-2} on segregating T_1 transgenic plants resulted in a segregation ratio for affected to unaffected plants of close to 3:1. Due to sizeable variation in shoot length of both non-transgenic and transgenic plants after treatment with a concentration >250 μg m^{-2}, a concentration of 100 μg m^{-2} was used in later experiments. The phenotypic effects of treatment of R7402 on T_1 transgenic and non-transgenic plants are shown in Figure 2. Plants sensitive to R7402 did not completely recover; shoots remained short-

Table 1. Germination frequency of transgenic T_2 (line 24B-1) and non-transgenic embryos on medium containing 5-fluorocytosine (5-FC)

5-FC concentration[a]	Number of plants tested		Germination frequency[b]	
	Non-transgenic	Transgenic	Non-transgenic	Transgenic
0	200	171	97.0 ± 2.1	95.0 ± 1.9
1	200	200	96.5 ± 1.9	52.0 ± 8.7
2	200	200	96.5 ± 1.8	29.5 ± 7.5
3	200	182	90.5 ± 1.8	22.0 ± 6.1
4	200	150	56.0 ± 12.4	16.0 ± 8.9

[a](mg/ml); [b]Values are % ± standard deviation between repetitions.

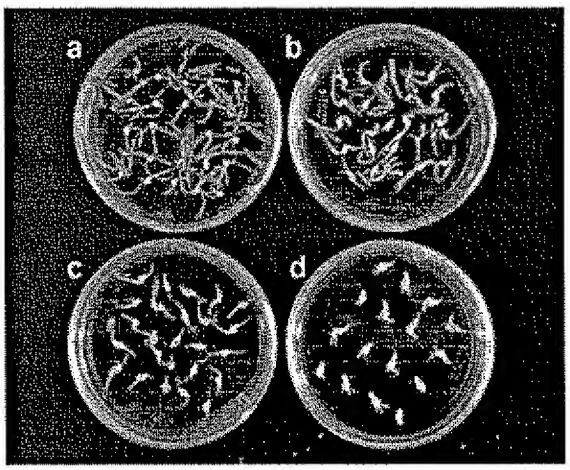

Figure 1. Negative selection using *codA*-containing transgenic and non-transgenic embryos.
Embryos from non-transgenic controls were transferred to medium without (a) and with (b) 2 mg ml^{-1} 5-FC, and embryos from transgenic plants were transferred to medium with 2 mg ml^{-1} 5-FC (c) and with 20 µg ml^{-1} 5-FU (d). The photograph was taken 7 days after plating of embryos.

er than those from non-transgenic plants but were capable of producing seed.

PCR analysis of segregating populations

To test the reliability of the negative selection schemes for screens of large populations, germinating embryos or transgenic plants transformed with either *codA* or *P450* genes were treated in several independent experiments with either 5-FC or R7402, respectively. Results of germination tests with embryos from *codA*-positive plants are summarized in Table 3. A total of 402 embryos from line 24B-1 and 467 embryos from line 25B-1 were tested, and segregation ratios of 305:97 and 361:106, respectively, for sensitive:unaffected were determined. The observed segregation ratio for both lines is close to the expected 3:1 ratio as confirmed by a χ^2 test with $\alpha = 0.05$. The embryos from a third line (26B-5) segregated in an aberrant manner (156:82, sensitive:resistant).

Five transgenic lines were tested for sensitivity to R7402. After treatment with R7402, T_1 plants from three transgenic lines segregated at the expected ratio of 3:1, sensitive: unaffected (Table 4). T_1 plants from line 4B-1 segregated in a ratio of 15:1; progeny of another line (3B-1) segregated in a ratio of 2:1.

From each experiment using either gene, a sample of 12 unaffected and four sensitive embryos or plants from a line were randomly chosen and analysed by PCR for the presence of the transgene. Since it is more important in a negative selection scheme to avoid or minimize the number of escapes from selection, a larger number of unaffected, compared to sensitive, plants was analysed. With respect to *codA*, a total of 96 of the 203 unaffected plants from line 24B-1 and 25B-1 were analysed; 93 embryos were PCR-negative while DNA from three embryos yielded the 500 bp internal *codA* fragment. DNA from 32 non-germinated embryos was tested for the presence of *codA*, and 31 yielded the expected PCR fragment. In the analysis of the plants putatively containing the cytochrome *P450* gene, 81 of 143 unaffected plants were tested by PCR; 79 were PCR-negative. The expected PCR product was amplified from DNA of 29 of the 30 sensitive plants tested.

Discussion

The use of negative selection genes as tools for various applications in genetic engineering has to date been restricted to dicotyledonous plant species. In our studies of transgenic barley, we used the bacterial genes *codA* for *in vitro* assays of germinating seeds and cytochrome *P450* for sensitivity tests of developing plants.

In earlier *in vitro* studies on seedlings of tobacco (Schlaman and Hooykaas, 1997; Stougaard, 1993), *Arabidopsis* (Kobayashi *et al.*, 1995; Perera *et al.*, 1993) and *Lotus japonicus* (Stougaard, 1993), a concentration

Table 2. Influence of R7402 on shoot development of transgenic T1 and non-transgenic plants

R7402 concentration[a]	Non-transgenic plants		Transgenic plants				
			Affected plants		Unaffected plants		
	n	Shoot length[b]	*n*	Shoot length[b]	*n*	Shoot length[b]	Ratio[c]
0	35	20.0 ± 0.85	0	–	39	19.9 ± 0.96	–
25	35	19.7 ± 1.10	0	–	38	19.5 ± 1.13	–
50	38	19.2 ± 1.21	31	13.0 ± 1.31	10	19.8 ± 0.92	31:10
100	39	18.0 ± 1.56	35	9.7 ± 1.37	12	18.8 ± 1.19	35:12
250	38	15.3 ± 2.24	33	6.4 ± 1.70	9	15.3 ± 1.46	33:9
500	40	11.7 ± 2.67	37	5.0 ± 1.33	10	13.0 ± 1.11	37:10
1000	37	8.7 ± 2.05	29	4.3 ± 1.45	9	9.0 ± 1.3	29:9

[a]($\mu g\ m^{-2}$); [b]Values are cm ± standard deviation between. repetitions; [c]affected:unaffected.

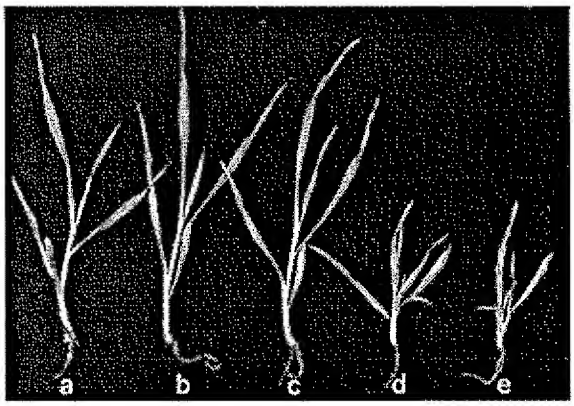

Figure 2. Effect of R7402 on *P450*-positive and control plants.
Seven-day-old seedlings were sprayed with selective agent R7402; the photograph was taken 21 days after the treatment. Order of plants (left to right): (a) non-transgenic plant sprayed with solvent alone; (b) non-transgenic plant sprayed with 100 $\mu g\ m^{-2}$ R7402; (c) non-transgenic T_1 segregant sprayed with 100 $\mu g\ m^{-2}$ R7402; (d); (e) two transgenic T_1 segregants sprayed with 100 $\mu g\ m^{-2}$ R7402.

of between 0.25 and 1 $mg\ ml^{-1}$ of 5-FC was sufficient to distinguish between *codA*-positive and *codA*-negative seedlings. These concentrations did not affect the growth and development of non-transgenic plants. In germination tests on embryos from *codA*-positive barley plants in this study, 5-FC concentrations of between 2 and 4 $mg\ ml^{-1}$ were necessary to obtain reliable selection for *codA* expression driven by the *Act1* promoter; however, a concentration of 4 $mg\ ml^{-1}$ 5-FC affected the germination and development of non-transgenic plants. PCR analyses of plant tissue from unaffected and sensitive germinating embryos showed that the expression of cytosine deaminase driven by the rice *Act1* promoter and first intron is sufficient in barley to distinguish between *codA*-negative and -positive plants in segregating populations. Similar reliability was observed using the cytochrome *P450* gene as a negative selection marker, after optimizing selection conditions to distinguish between plants with and without the *P450* gene. Differences between expressing and non-expressing plants were most striking when R7402 concentrations were between 100 and 250 $\mu g\ m^{-2}$; higher concentrations affected the development of control and negative segregant plants.

The high standard deviation observed (Table 1) and the escapes from negative selection are probably due to variable expression levels of the selectable marker genes and inherent plant to plant variation in sensitivity. This variability can affect the efficiency and reliability of the selection scheme (Czakó *et al.*, 1995). Escapes from negative selection using *codA* may also be due to differences in sensitivity depending on the developmental state of the embryos. Since the frequency of escapes is low (2.1% for *codA* and 3.7% for *P450*), both negative selectable markers can be used reliably for selection. The technical difficulties of testing uniformly sized embryos for *codA* expression, however, make this approach less practical and less amenable for large-scale screening. The possible compromising effects of transgene inactivation in scoring advanced-generation plants (Renckens *et al.*, 1992) will have to be addressed if the system is to achieve maximum utility.

Despite differences between the regulatory sequences of monocotyledonous and dicotyledonous *rbc*S promoters (Schaeffner and Sheen, 1991), the functionality of dicot *rbc*S promoters in monocots has been demonstrated in transgenic rice (Kyozuka *et al.*, 1993). In our experiments, the expression level of the dicot *rbc*S promoter was sufficient to lead to the dealkylation of the sulfonyl urea compound R7402. In plants, the Rubisco protein requires redox donors from the chloroplast to carry out its monooxygenase function (O'Keefe *et al.*, 1994). Therefore, the

Table 3. Germination test on 5-FC and PCR analysis of embryos from *codA*-containing plants

Plant	Germination ratio[a]	PCR analysis					
		Sensitive embryos			Resistant embryos		
		Number tested	Number positive	Number negative	Number tested	Number positive	Number negative
Non-transgenic	4:315	4	0	4	6	0	6
24B-1	305:97	16	16	0	48	1	47
25B-1	361:106	16	16	0	48	1	47

[a]Sensitive:resistant.

Table 4. Sensitivity to R7402 and PCR analysis of progeny from *P450*-containing plants

Plant	Segregation ratio[a]	PCR analysis					
		Affected plants			Unaffected plants		
		Number tested	Number positive	Number negative	Number tested	Number positive	Number negative
Non-transgenic	2:129	2	0	2	6	0	6
3A-1	91:39	6	6	0	20	0	20
3A-2	88:32	6	6	0	20	1	19
3A-3	63:26	6	5	1	16	0	16
3B-1	70:35	6	6	0	14	0	14
4B-1	110:11	6	6	0	11	1	10

[a]Affected:unaffected.

Petunia Rubisco chloroplast transit peptide was used to target cytochrome P450 to the chloroplast in tobacco (O'Keefe *et al.*, 1994). In our studies, the same transit peptide sequence was used to target the protein to the chloroplast in a monocot species. Its use resulted in functional activity of the mono-oxygenase as evidenced by its ability to inhibit the development of *P450*-positive plants.

The results of our studies indicate that both bacterial negative selectable genes, *codA* and the cytochrome *P450* gene, can be used effectively in cereals. The use of the *codA* gene is restricted to *in vitro* tests of functionality in embryos of a specific developmental state. In contrast, the *P450* gene can be used for selection of plants in the greenhouse or field, enabling screening of large numbers of individual plants without labour-intensive *in vitro* manipulation. Difficulties in recovering *codA*-expressing plants after selection with 5-FC also restrict this approach to situations where the survival of positive plants is not necessary. Negative selection systems such as these can be powerful tools for transgenic cereals, for example to select against undesirable linked genes, e.g. *Ac transposase*, to dissect signal transduction pathways and to conduct functional genomics studies.

Experimental procedures

Construction of vectors

Plasmid pAHC20 (Christensen and Quail, 1996), containing the *Streptomyces hygroscopicus* phosphinothricin acetyl transferase gene (*bar*) under the control of the maize *Ubi1* promoter and the first intron, was used in selection experiments with bialaphos. Figure 3 shows diagrams of all other plasmids used in this study. All DNA modifications were carried out according to standard protocols (Sambrook *et al.*, 1989). The *codA* coding region and CaMV 35S 3′ terminator was cloned as a 1.3 kbp *Hin*dIII–*Eco*RI fragment from pNE3 (kind gift of Dr J. Stougaard) under transcriptional control of the *Act1* promoter and the first intron from rice (McElroy *et al.*, 1990) into a pUC28- or pBS-derived plasmid. The resulting vectors pUC-codA-Act-AcAc and pBS-codA-Act-UbiAc also contain the *Ac* transposase coding region and *nos* terminator sequence derived from pTps (Wirtz *et al.*, 1997) as a 3.6 kbp *Sma*I–*Bgl*II fragment under the control of either a 323 bp *Sac*I–*Bgl*II *Ac* transposase promoter fragment from plasmid pSLJ721 (Scofield *et al.*, 1992) or the *Ubi* promoter and first intron from maize (Christensen and Quail, 1996).

For the construct carrying the *P450* gene, a 4.6 kb *Eco*RI–*Bam*HI cassette containing the Rubisco promoter, 5′ untranslated leader

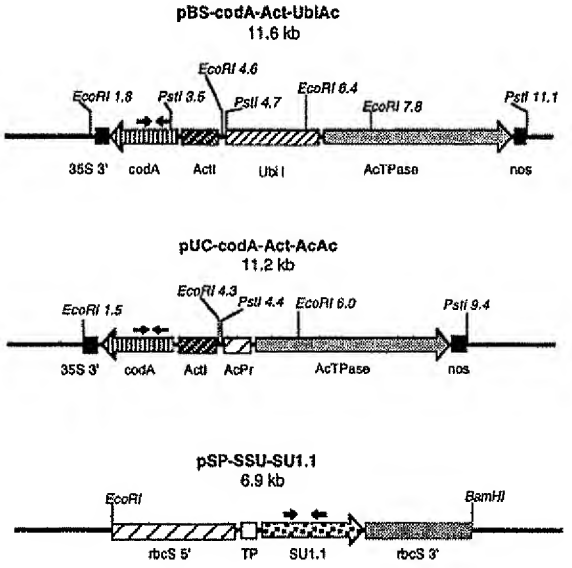

Figure 3. Schematic representation of plasmids used.
The *codA* gene under control of the rice actin promoter (*Act1*) and first intron and the 35S terminator were cloned together with the activator transposase (AcTPase) ORF under control of its putative promoter sequence (AcPr) and the *nos* terminator (pUC-codA-Act1-AcAc) or the *Ubi1* promoter and first intron from maize and the *nos* terminator (pBS-codA-Act-UbiAc) Plasmid pSP-SSU-SU1.1 contains the Rubisco small subunit promoter (rbcS 5') from *Petunia hybrida*, the coding sequence for the chloroplast transit peptide (TP), the Rubisco poly(A) signal (rbcS3') and the *P450* coding region (SU1.1) from *S. griseolus*. Restriction sites used for DNA hybridization analysis are indicated. Arrows indicate the amplified regions in the PCR analysis.

and the coding region for the Rubisco transit peptide for chloroplast targeting from *Petunia hybrida* (Dean *et al.*, 1989), the complete *P450* coding region from *S. griseolus* (Omer *et al.*, 1990) and the Rubisco poly(A) signal sequence (O'Keefe *et al.*, 1994) were cloned into the 2.46 kb cloning vector pSP72 (Promega, Madison, Wisconsin, USA) resulting in the plasmid pSP-SSU-SU1.1. Additional details regarding the vectors and plasmid maps are available upon request from Dr Peggy G. Lemaux.

Plant transformation and regeneration

Cultivation, transformation via microprojectile bombardment and selection of transformed barley scutellar tissue (*Hordeum vulgare* L. cv Golden Promise) was performed as described (Lemaux *et al.*, 1996). Scutella were co-transformed with equimolar amounts of either pAHC20 and pSP-SSU-SU1.1 , pAHC20 and pUC-codA-Act-AcAc or pAHC20 and pBS-codA-Act-UbiAc. T_0 plants were regenerated from stably transformed callus following the protocol of Cho *et al.* (1998). Phenotypically normal T_0 plants were analysed by DNA gel-blot hybridization to determine the presence and copy number of the transgenes. Plants with one to three intact copies of the expression cassette were used for further studies. The progeny resulting from self-pollination were analysed by PCR. PCR-positive T_1 and T_2 plants were used for the *codA* and *P450* gene activity tests.

5-fluorocytosine assay for codA *gene expression*

Spikes of *codA*-positive plants, as determined by PCR, were sterilized 14–20 days post-anthesis. Immature embryos (2–2.5 mm) were isolated as previously described (Wan and Lemaux, 1994) and transferred embryo axis-side down to germination medium (MS salts: Murashige and Skoog, 1962), solidified with 8 g l^{-1} agar (Difco) at pH 5.8), and containing 15 g l^{-1} sucrose and either 5-fluorocytosine (5-FC) or 5-fluorouracil (5-FU). 5-FC and 5-FU were dissolved in 10 mM 2-(*N*-morpholino) ethanesulphonic acid (MES) (pH 5.8), filter-sterilized and added after autoclaving. To determine immature embryo sensitivity, 5-FC was used at concentrations of between 1 and 4 mg ml^{-1} (J. Louwerse, unpublished data); in later experiments the concentration of 5-FC used was 2 mg ml^{-1}. Germinated and non-germinated embryos were counted on day 7. To study the cytotoxic effect of the metabolite 5-FU, the compound was used at a concentration of 0.02 mg ml^{-1}. Immature embryos from tissue culture-derived, non-transgenic plants were used as controls in all experiments.

R7402 herbicide assay for P450 *gene expression*

T_1 seeds of *P450*-positive T_0 plants, as confirmed by PCR analysis, were germinated and plants grown in potting soil in flats under greenhouse conditions (14 h light/10 h dark, 15–18°C, natural and supplemental light levels at 700–1000 µmol m^{-2} sec^{-2}). The herbicide, R7402 (2-methylethyl-2,3-dihydro-*N*-[(4,6-dimethoxypyrimidin-2-yl) aminocarbonyl]-1,2-benzoisothiazole-7-sulfonamide-1,1-dioxide, Du Pont de Nemours, Wilmington, Delaware, USA), was dissolved in a mixture of 90.2% acetone (v/v), 4.8% glycerol (v/v), 4.8% water (v/v) and 0.2% Tween-20 (v/v) and plants were sprayed 10 days post-germination. Flats (0.15 m^2) were sprayed with a total volume of 25 ml containing the appropriate dose of herbicide in three intervals using a hand-held atomizer. To determine sensitivity, concentrations of R7402 equivalent to 25, 50, 100, 500 and 1000 µg m^{-2} were used. An R7402 concentration of 7.5 µg/25 ml (equivalent to 100 µg m^{-2}) was used in later experiments. As an indicator of growth inhibition, shoot length was measured 3 weeks after herbicide application. Tissue culture-derived, non-transgenic plants were used as controls in all experiments.

PCR analyses

Genomic DNA was isolated from leaf tissue or from germinated embryos (Cone, 1989). PCR reactions were performed in a total volume of 50 µl containing 1× PCR buffer (Promega, Madison, Wisconsin, USA), 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1 µM primer, 1% DMSO, 2.5 U Taq DNA polymerase (Promega) and 0.5 µg of genomic DNA. Primers used for amplification of the *codA* coding region were *codA*-1: 5'-GCA AAT CGT CGC CTT CCC TCA GGA-3' and codA-2: 5'-AAG ACA TCA TCG TGA CCA AAG CAG-3', which yielded a 500 bp fragment (Figure 3). For detection of the cytochrome *P450* gene, the primer pair SU-1: 5'-TGC CCT ACG CCG ACC ACG AGT TCT-3' and SU-2: 5'-CCT TGC CCC TCT CCC CCG TCA CCA-3' was used to amplify a 750 bp fragment from the coding region (Figure 3). Both PCR reactions were performed with an initial denaturation at 94°C for 2 min followed by 35 cycles of 45 sec at 65°C and 60 sec at 72°C, and a final extension at 72°C for 5 min. PCR products were analysed by gel electrophoresis on 1.1% agarose gels.

Acknowledgements

The authors would like to thank Drs Uwe Wirtz and Barbara Baker for plasmid pTps, Dr Jens Stougaard for plasmid pNE3, Dr

Jonathan Jones for the gift of plasmid pSLJ721 and Dr Dan O'Keefe for the *P450* gene and R7402 compound. T.K. was supported by a postdoctoral fellowship of the Deutsche Forschungsgemeinschaft (Ko1791/1-2). D.M. and J.L. were supported by funding from the North American Barley Genome Mapping Project and USDA NRI competitive grant number 9500682. R.W.-C. and P.G.L. were supported by the USDA Cooperative Extension Service through the University of California.

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Plant Molecular Biology Reporter 18: 227–233, 2000

Commentary

The *tms2* Gene as a Negative Selection Marker in Rice

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Abstract. A conditional negative selection marker is essential for high throughput insertional mutagenesis with any two-element transposon tagging system. The *tms2* gene encodes indoleacetic acid hydrolase (IAAH) which converts naphthaleneacetamide (NAM) to the potent auxin naphthaleneacetic acid, a phytotoxic derivative. This gene, under the control of the nanopine synthase gene 2 promoter from *Agrobacterium tumefaciens* and exogenously applied NAM, have been used effectively as a negative selector in *Ac/Ds* insertional mutagenesis of *Arabidopsis thaliana* (Sundaresan et al., 1995). In this study we show that *tms2* can also be used as a negative selector in rice. T_1 transgenic seedlings expressing this *tms2* gene under the control of the *mas2'* promoter showed significant reduction in shoot and root growth in the presence of 5-10 μM NAM under specified growth conditions compared to plants not containing this gene.

Key words: IAAH, negative selector, rice, *tms2* gene

Abbreviations: *Ac*, activator; *Ds*, dissociation; IAAH, indoleacetic acid hydrolase; NAA, naphthaleneacetic acid; NAM, naphthaleneacetamide.

Introduction

Introduction of a conditional negative selection marker gene linked to a transgene greatly facilitates counter selection for progeny devoid of transgenes. Conversion of an externally provided specific substrate into its phytotoxic derivative by the marker gene encoded enzyme enables this counter selection. The *tms2* gene was the first conditional selective marker gene to be used in tobacco (Depicker et al., 1988) and in *Arabidopsis* (Karlin-Neuman et al., 1991). Indoleacetic acid hydrolase (IAAH) encoded by the *tms2* gene confers sensitivity of plants to naphthaleneacetamide (NAM) because IAAH converts NAM to the potent auxin naphthaleneacetic acid (NAA) which inhibits seedling growth. Other conditional markers proven to be effective in dicots are *aux2* in cabbage (Beclin et al., 1993), the HSV-tk gene in tobacco (Czako and Marton, 1994), a bacterial cytochrome *P450* mono-oxygenase gene in tobacco (O'Keefe et al., 1994) and *Arabidopsis*

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(Tissier et al., 1999), and *codA* in *Arabidopsis* (Kobayashi et al., 1995) and tobacco (Schlaman and Hooykaas, 1997). So far, the cytochrome *P450* (the product of which catalyses the dealkylation of a sulfonylurea compound, R7402 into its cytotoxic metabolite) and *codA* (whose product cytosine deaminase converts the non-toxic 5-fluorocytosine into phytotoxic 5-fluorouracil) are the only genes to have been used as conditional negative selectors in monocots. Both have been proven to be effective in barley (Koprek et al., 1999). The only gene used in rice so far is the cytochrome *P450* (Chin et al., 1999). In this study we report that the *tms2* gene is an effective conditional negative selection marker for rice.

Materials and Methods

Gene construction and rice transformation

A *Cla* I partially-digested fragment from plasmid pWS42 (gift from V. Sunderesan) comprising *tms2* was first cloned into Bluescript KS^+ and an *Sst* I-endfilled-*Apa* I fragment was then cloned into *Hin*d III-endfilled-*Apa* I digested pMNRTT101 (unpublished), which is a derivative of the binary vector pWBVec8 (Wang et al., 1997), containing an intron interrupted *hph* gene driven by CaMV35S promoter. The resulting binary construct pMNRTT122 has the architecture RB –*mas* 2' promoter-*tms2*-polyA-CaMV35S promoter-*hph-nos* terminator- LB (Figure 1A). This construct was introduced into *Agrobacterium* strain AGL1 according to Upadhyaya et al. (2000). *Agrobacterium*-mediated transformation of rice cv. Nipponbare was performed and primary transgenic (T_0) lines were analyzed according to Upadhyaya et al. (2000).

PCR and Southern blot analyses

Genomic DNA was isolated from plants as described by Draper and Scott (1988). T_1 progeny plants were screened for the presence of the selectable marker gene by polymerase chain reaction (PCR). Primers were designed using the PRIME program of GCG (Devereux et al., 1984) based on the published sequences. Primers used were: MN35S-3 (5'-GGGATGACGCACAATCCC-3'), MNHPH5-180 (5'-GATCTTTGTAGAAACCATCGGC-3'). Southern blot analysis was performed on *Bam*H I digested genomic DNA using a radioactively-labelled *tms2* gene sequence (see Figure 1).

NAM sensitivity assay

A 10 mM stock of α-napthaleneacetamide (NAM, Sigma Chemical Co., USA) was prepared in 95% ethanol. Ten seeds each from the T_0 plants and a wild type plant were surface sterilized (70% ethyl alcohol for 90 s followed by 1.25% bleach for 20 min and wash with water), placed in plastic pots containing 0.5 strength MS complete solid medium (Murashige and Skoog, 1962) with five different levels of NAM (0, 5, 10, 25 and 100 μM), kept in the dark for 3 d (26°C) and then shifted to light (22-26°C with 16 h light of 20-40 μmol m^{-2} s^{-1}). Measurements of root and shoot lengths were taken after 12 d. Seedlings were then replanted in Jiffy pots containing a mixture of soil, perlite, sand and peat moss

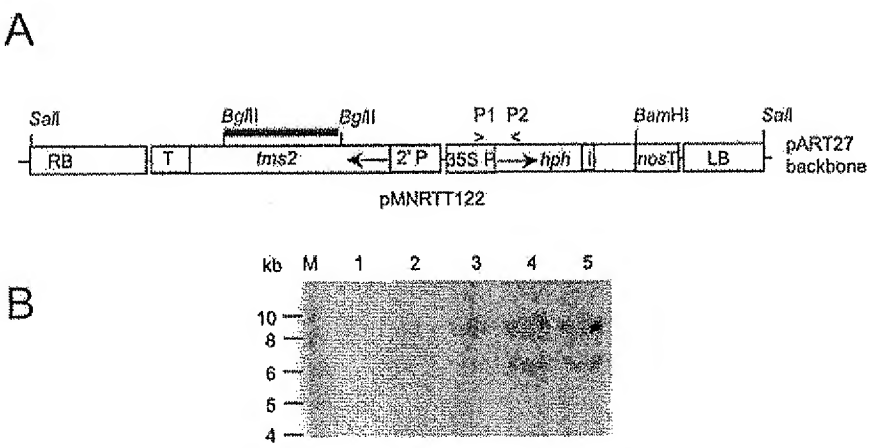

Figure 1. Schematic representation of the binary vector construct used (A) and demonstration by Southern blot hybridization of the presence of *tms2* transgene in primary transformants (B). The *tms2* gene under the control of *mas* gene 2' promoter and its own terminator was inserted into the binary vector pMNRTT101 to produce pMNRTT122. An intron interrupted *hph* gene under the control of CaMV35S promoter and nos terminator served as the selectable marker gene in *Agrobacterium*-mediated transformation of rice (cv. Nipponbare). Genomic DNA isolated from putative transgenic plants was digested with *Bam*H I and hybridized with the *tms2* gene probe (indicated by thick line in A). Binding sites of the primers used in segregation analysis are indicated (P1, P2).

(50:25:15:10 by volume) and kept in a mist chamber in a naturally illuminated glasshouse with 28°C day and 22°C night temperatures.

Results and Discussion

The aim of the present study was to find out whether the *tms2* gene could be used as an effective conditional negative selection marker for high throughput insertional mutagenesis in rice. Our efforts to use a CaMV35S promoter driven *codA* gene for this purpose were not successful (data not shown) even though the *Act1* promoter driven *codA* has recently been shown to work in barley (Koprek et al., 1999). The only other conditional negative selector effectively used in rice has been a bacterial cytochrome *P450* mono-oxygenase gene under the control of Rubisco promoter and chloroplast targeting signals (Chin et al., 1999). This gene has previously been shown to work in tobacco (O'Keefe et al., 1994), *Arabidopsis* (Tissier et al., 1999) and barley (Koprek et al., 1999) with a sulfonylurea compound, R7402 as substrate. The *tms2* gene (containing its own terminator sequences) fused to the manopine synthase (*mas*) 2' gene promoter was used by Sunderesan et al. (1995) as a conditional negative selector in their transposon trapping work in *Arabidopsis thaliana*. We have used the same 2' promoter-*tms2* gene cassette in the binary vector pMNRTT122 (Figure 1A) that contains an intron-interrupted *hph* gene driven by a CaMV35S promoter (Wang et al., 1997). Five out of six plants regenerated from hygromycin resistant rice calli, produced by *Agrobacterium*-mediated transformation, were fertile. Southern blot analysis of *Bam*H I-digested genomic DNA from these T_0 plants using radioactively-labelled

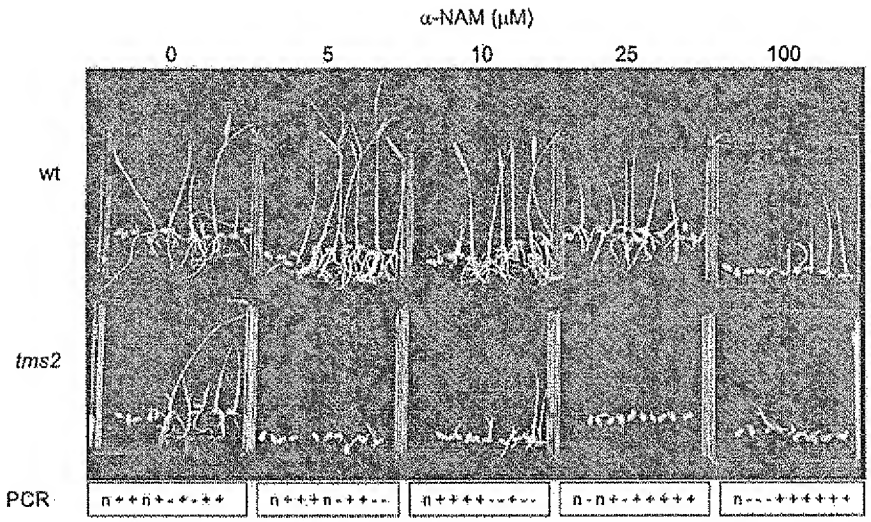

Figure 2. Growth inhibition of T_1 seedlings of *tms2* transgenic rice. Surface sterilized transgenic (*tms2*) and wild type (wt) seeds were germinated in 0.5 strength MS solid medium containing different levels of NAM in the dark for 3 d and transferred to light. The photograph was taken 9 d after transfer to light. Presence (+) or absence (-) of the *tms2* transgene in these plants were confirmed by PCR. Plants not analyzed by PCR are indicated (n).

tms2 gene sequence as a probe revealed two hybridizing bands present in each transgenic plant (Figure 1B). The similarity of the hybridization patterns suggests that all plants were from a single transformation event. Germination testing of T1 seeds in the presence of hygromycin revealed a 3:1 segregation of the *hph* gene in all, also suggesting a single locus transformation event. All T1 seeds from T_0 plants were therefore pooled as progeny of a single transgenic line for IAAH sensitivity assay.

The substrate α-naphthaleneacetamide (NAM) was used according to Karlin-Neumann et al. (1991). NAM at 5 μM concentration affected root and shoot growth of transgenic seedlings (Figure 2). This concentration of NAM also appeared to cause a growth promoting effect on wild type seedlings. We can distinguish between *tms2* transgenic seedlings expressing IAAH and wild type or null seedlings at 5 μM NAM even though transgenic seedlings had reduced vigor compared to wild type seedlings. At 10 μM NAM wild type (non transgenic) seedlings had similar growth to untreated plants while the transgenic seedlings showed drastic growth inhibition with this concentration of NAM. Growth inhibition was observed in control seedlings as well at 25 μM NAM and at 100 μM NAM the effect was very severe. PCR analyses of DNA isolated from seedlings recovered after removing the negative selection revealed segregation of the *tms2* transgene and seedlings with minimum growth inhibition under NAM selection were nulls. Figure 2 summarizes the effect of different levels of NAM on shoot and root growth of *tms2* transgenic seedlings compared to wild type seedlings. Thus, we

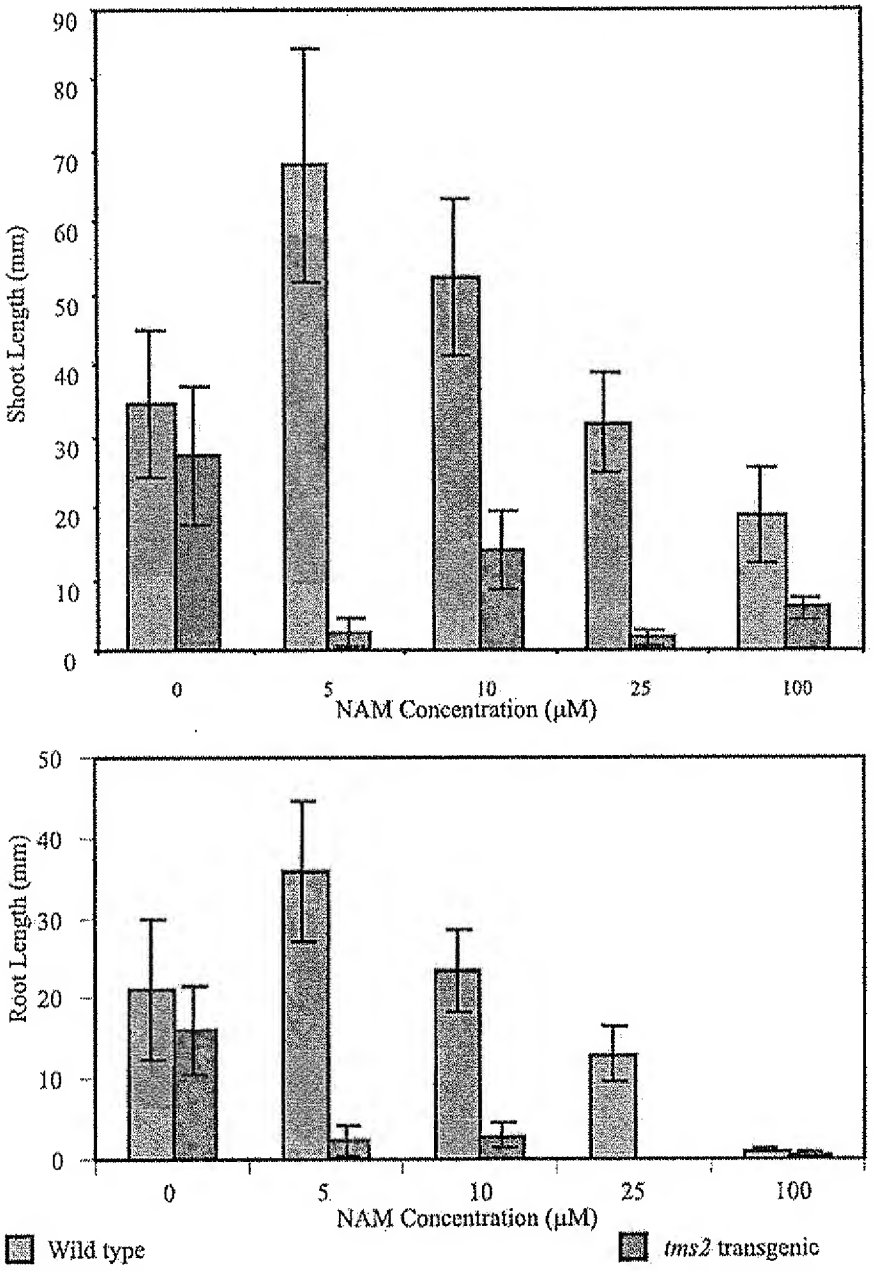

Figure 3. Effect of NAM on root and shoot growth of *tms2*-containing transgenic and wild type seedlings. Bar diagrams showing the average shoot and root lengths in mm (10 seeds) of 12 day-old seedlings (including ungerminated seeds) and the standard error of means.

demonstrate that *tms2* gene can be used as an effective conditional negative selection marker in rice at NAM concentrations 5-25 μM.

Acknowledgements

The plasmid pWS42 containing the *tms2* gene was kindly provided by Dr. V. Sundaresan, Institute of Molecular Agrobiology, Singapore.

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The Plant Journal (1999) 18(5), 571–576

TECHNICAL ADVANCE

A bacterial haloalkane dehalogenase gene as a negative selectable marker in *Arabidopsis*

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Summary

The *dhlA* gene of *Xanthobacter autotrophicus* GJ10 encodes a dehalogenase which hydrolyzes dihaloalkanes, such as 1,2-dichloroethane (DCE), to a halogenated alcohol and an inorganic halide (Janssen *et al.*, 1994, *Annu. Rev. Microbiol.* 48, 163–191). In *Xanthobacter*, these alcohols are further catabolized by alcohol and aldehyde dehydrogenase activities, and by the product of the *dhlB* gene to a second halide and a hydroxyacid. The intermediate halogenated alcohols and, in particular, the aldehydes are more toxic than the haloalkane substrates or the pathway products. We show here that plants, including *Arabidopsis*, tobacco, oil seed rape and rice, do not express detectable haloalkane dehalogenase activities, and that wild-type *Arabidopsis* grows in the presence of DCE. In contrast, DCE applied as a volatile can be used to select on plates or in soil transgenic *Arabidopsis* which express *dhlA*. The *dhlA* marker therefore provides haloalkane dehalogenase reporter activity and substrate dependent negative selection in transgenic plants.

Introduction

Negative selectable marker genes generally encode enzymes which metabolize a specific substrate to a cytotoxic product. For example, transgenic plants expressing *E. coli codA*, which deaminates 5-fluorocytosine (5-FC) to cytotoxic 5-fluorouracil, are unable to grow on media containing 5-FC (Stougaard, 1993). Such substrate-dependent negative markers can be used in genetic techniques including targeted recombination, tissue-specific cell death, and mutant screens. For example, *Agrobacterium tms2*, which hydrolyzes indole-3-acetamide to the auxin indole-3-acetic acid, is used as a marker in a maize *Ds* transposon system to select for transposition events that are unlinked to the donor loci (Sundaresan *et al.*, 1995). Expression in tapetal cells of *S. griseolus* $P450_{SU1}$, which converts the sufonylurea R70402 to a potent herbicide, can be used to produce male sterility (O'Keefe *et al.*, 1994). A constitutively expressed nitrate reductase gene can be used in chlorate resistant selections of mutants impaired in nitrogen uptake, metabolism or their regulation (Nussaume *et al.*, 1991). Lastly, *Arabidopsis* alcohol dehydrogenase, which hydrolyzes allyl alcohol to the toxic aldehyde acrolein, has been used in a fusion genetic screen to select trans-acting regulatory mutants (López-Juez *et al.*, 1998).

A novel substrate-dependent negative selection marker based on the *dhlA* gene of *Xanthobacter autotrophicus* GJ10 is presented here. The *dhlA* gene encodes a single chain, co-factor independent dehalogenase which hydrolyzes haloalkanes, such as 1,2-dichloroethane (DCE), to a halogenated alcohol and an inorganic halide (Janssen *et al.*, 1994). The X-ray structure and catalytic mechanism of the enzyme have been determined (Schanstra *et al.*, 1996; Verschueren *et al.*, 1993) In *Xanthobacter*, the alcohols produced are further catabolized by alcohol and aldehyde dehydrogenase (ADH and ALDH) activities, and by the *dhlB* haloalkanoic acid dehalogenase to a second halide and a hydroxyacid (Figure 1). The intermediate halogenated alcohols and particularly the aldehydes are far more toxic than the haloalkane substrates or the final products of the pathway.

Two potential uses of the *dhlA* encoded haloalkane dehalogenase (DhlA) can be envisioned in transgenic plants. First, because plant ADH and ALDH activities are normally low in roots and leaves (op den Camp and Kuhlemeier, 1997), plants expressing high levels of DhlA haloalkane dehalogenase and supplied with DCE or similar substrates might accumulate toxic levels of the product alcohols or aldehydes. In this case, and as shown here, *dhlA* can be used as a substrate-dependent negative marker. Second, various DhlA substrates are important environmental pollutants in soil and groundwater, including the bulk synthetic chemical DCE and the fumigants dichloropropene and DBE. Transgenic plants capable of fully degrading such compounds via the activity of DhlA

Received 14 January 1999; revised 16 April 1999; accepted 19 April 1999.
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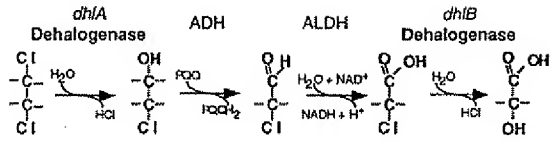

Figure 1. Catabolic pathway of 1,2-dichloroethane in *X. autotrophicus* (from Janssen *et al.*, 1994).
ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; PQQ, ADH pyrroloquinoline quinone cofactor; NAD, ALDH nicotinamide adenine dinucleotide co-factor.

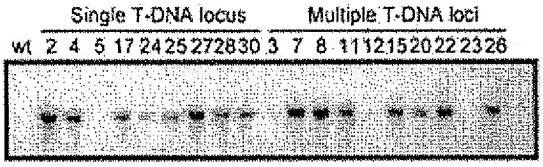

Figure 2. Northern blot analysis of *dhlA* RNA in *Arabidopsis* lines transformed with the 2×35S/*dhlA* construct.
10 μg total RNA was loaded per lane. Line numbers (top) are grouped as containing single or multiple T-DNA loci (T2 seed exhibiting 3:1 KAN^R or >3:1 KAN^R). Wt. control is untransformed Ecotype Ws. The filter was exposed for 6 h.

could therefore be useful in bioremediation of contaminated sites (Janssen *et al.*, 1994). As a first step, we show here that DhlA dehalogenase can be expressed in *Arabidopsis*, and that the *dhlA* gene can be used as a negative marker in plants.

Results

Production of transgenic Arabidopsis *expressing the* dhlA *gene*

The *dhlA* coding region was isolated as a linkered PCR fragment from plasmid pKLA (Schanstra *et al.*, 1993) and directionally cloned between the double CaMV 35S promoter and pea SSU E9 terminator in pKYLX71:35S2 (Schardl *et al.*, 1987). The resulting construct was introduced into *Arabidopsis* and transgenic lines selected on kanamycin (KAN^R) were allowed to self (Bechtold *et al.*, 1993). KAN^R T2 plants among progeny exhibiting 3:1 KAN^R segregation (single integration site) or >3:1 KAN^R segregation (multiple insertions) were allowed to self-pollinate and the seed from T3 lines homozygous for at least one T-DNA insertion locus (100% KAN^R progeny) was collected. No abnormal phenotypic effects under normal growth conditions were noted for any transgenic line.

PCR on leaf DNA (not shown) and Northern blotting were used to identify kanamycin-resistant transgenic lines expressing *dhlA* RNA (Figure 2). The levels of expression among lines containing single and multiple T-DNA loci

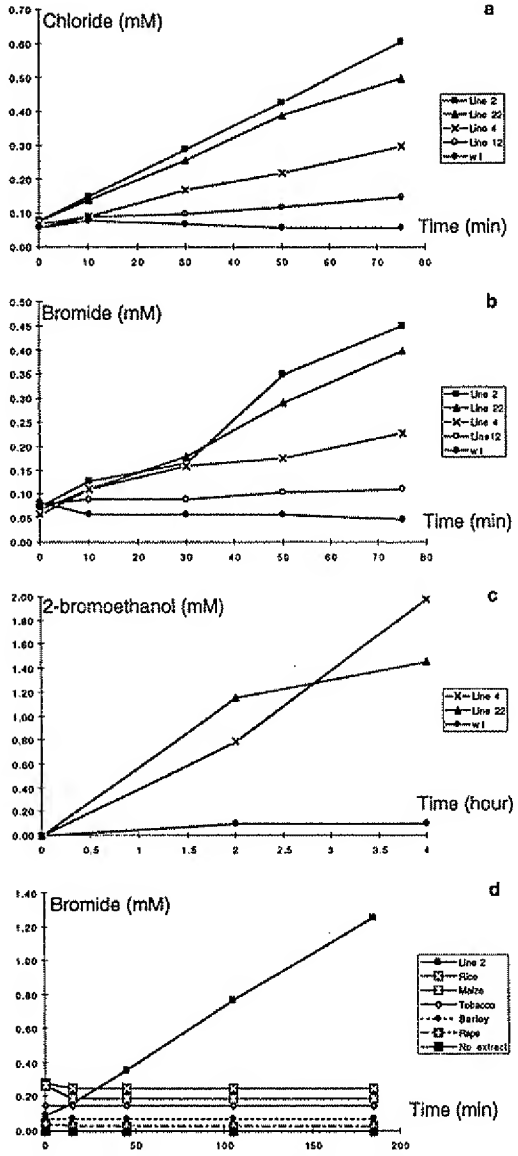

Figure 3. Haloalkane dehalogenase activities of transgenic *Arabidopsis* and other plants.
Halide production from (a) 1,2-dichloroethane and (b) 1,2-dibromoethane by extracts of wild-type and transgenic *Arabidopsis* lines as noted. (c) Production of 2-bromoethanol from 1,2-dibromoethane by *Arabidopsis* extracts. (d) Halide production from 1,2-dibromoethane by different plant species.

varied, presumably due to copy number and position effects.

Measurements of haloalkane dehalogenase activities in transgenic Arabidopsis *and in other plants*

Lines 2, 4, 12 and 22 accumulating relatively high levels of *dhlA* RNA were tested for their ability to dehalogenate the

model substrates DCE and DBE (Figure 3a,b). Dehalogenase activity was detected in the transgenic lines but not in the untransformed control, and the levels of enzyme activity in the transgenic lines correlated well with the levels of *dhlA* RNA detected in them by Northern blotting (Figure 2). These results demonstrate that active DhlA dehalogenase can be expressed in *Arabidopsis*.

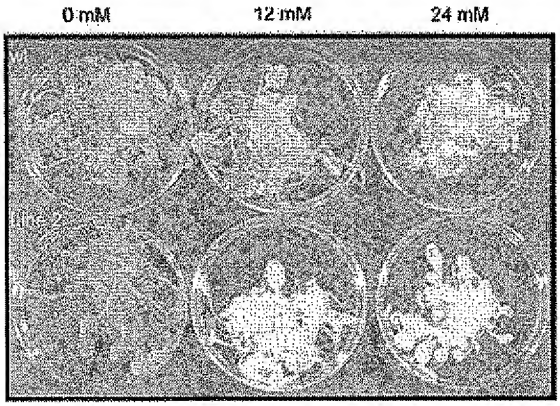

Figure 4. Negative selection using DCE.
Phenotypic effects of DCE added to liquid cultured, 2-week-old wild-type (upper row) or line 2 *dhlA* transgenic (lower row) plantlets for 48 h.

Table 1 shows that the specific activities for DCE of extracts of lines 2, 4, 12 and 22 range from 34 mU to 228 mU mg^{-1} extract protein. We calculate that in the highest expressing line 2, DhlA dehalogenase may represent 2% of total soluble extract protein, based upon the specific activity of the purified, recombinant enzyme expressed in *E. coli* (Schanstra *et al.*, 1993). The ratio of the activities with DBE and DCE of the protein expressed in plants corresponds well with the activity ratios of the protein purified from *E. coli*. Most modifications of the protein result in a decrease of the ratio of DCE versus DBE

Table 1. Dehalogenating activities of transgenic *Arabidopsis* for 1,2-dibromoethane (DBE) and 1,2-dichloroethane (DCE)

Sample	Spec. act. for DCE (mU mg^{-1} protein)	Spec.act. for DBE (mU mg^{-1} protein)
Wild type	<5	<5
2	228	175
4	119	77
12	34	13
22	188	140

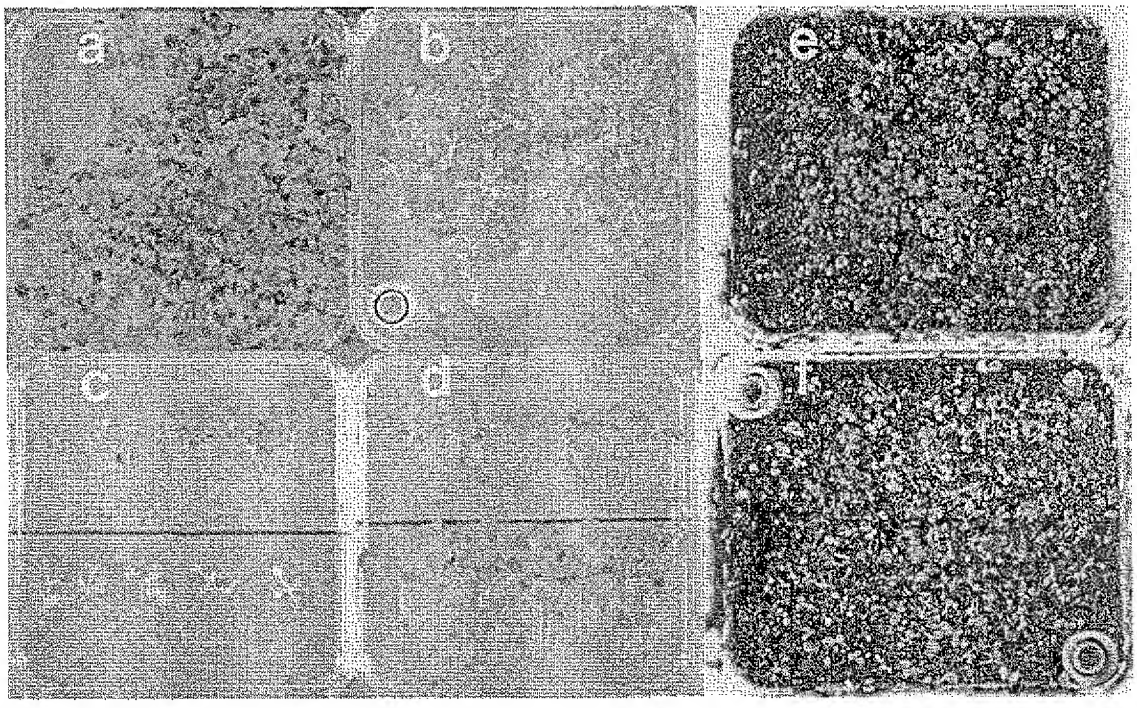

Figure 5. Reconstitution screen for negative selection using DCE.
(a) Phenotype after 16 days of growth on MS agar plates of a 1:25 mixture of untransformed, wild-type seed and seed of transgenic line 2. (b) The same mixture after 14 days of growth followed by 2 days of selection in the presence of 5 µl of volatile DCE added to a microtube cap. Plantlets selected in (b) as transgenic (upper row) or wild-type (lower row) were transferred to MS/Kan (c) or MS (d) plates and allowed to grow for an additional 6 days. Phenotypes of a 1:10 mixture of wild-type and transgenic line 2 seeds after 16 days in soil (e) and 14 days in soil followed 2 days of selection with 30 µl of DCE added to a microtube cap.

activity (Schanstra *et al.*, 1996). The fact that this ratio is roughly the same in *Arabidopsis* indicates that the protein is correctly folded and not deleteriously modified.

The formation of 2-bromoethanol from 1,2-dibromoethane was also followed in the high expressing lines 4 and 22 (Figure 3c). Rapid production of 2-bromoethanol was found which indicates that the expected hydrolytic dehalogenation occurs in these plants.

Extracts of *Arabidopsis*, maize, rape, tobacco and barley were tested for endogenous activities towards DBE (Figure 3d) and DCE (not shown). None of these extracts showed significant levels of activity on either substrate, indicating that there is normally no haloalkane dehalogenating activity present in these plants.

Negative selection using 1,2-dichloroethane of transgenic Arabidopsis *expressing* dhlA

The effect of different concentrations of DCE on the growth phenotypes of wild-type and *dhlA* expressing transgenic *Arabidopsis* was then examined. This showed that 12 mM DCE did not significantly effect the growth in liquid media of wild-type (Figure 4 upper) or plants transformed with an empty vector control (not shown). In contrast, this concentration of DCE caused severe bleaching of *dhlA* transgenic lines 2 and 12 (Figure 4 lower).

To examine whether the *dhlA* transgene could be used as a selectable marker in practical screening experiments, reconstitution assays were performed on plates and in soil with mixtures of wild-type and *dhlA* expressing transgenic plantlets. This showed that low concentrations of DCE (approximately 1.5–2.0 mM in air), added in a microtube cap as a volatile liquid to MS plates or phytatrays with soil, did not significantly effect the growth phenotype of wild-type plantlets but led to severe bleaching of *dhlA* transgenic plantlets (Figure 5a versus 5b and 5e versus 5f). The efficacy of the selection was demonstrated by transferring plantlets scored phenotypically as transgenic or wild-type to MS/KAN or MS plates (Figure 5c,d). This showed that plants selected as *dhlA* transgenics recovered normal growth on both MS/KAN and MS (Figure 5c,d, top row), while plants selected as wild-type grew well on MS but not on MS/KAN (Figure 5c versus 5d, lower row). In three experiments, only two out of 300 (< 1%) plants scored as DCE resistant proved to be not kanamycin resistant upon transfer to antibiotic plates and were therefore escapes.

Discussion

Expression of the bacterial DhlA dehalogenase from a constitutive promoter in *Arabidopsis* provides negative selection with millimolar concentrations of DCE. This selection system works well for plants growing both on plates and in soil. It is amenable to large scale screening because the DCE substrate is volatile and can be easily added in small volumes to closed containers at selected times during the growth of plants. Recovery of selected *dhlA* expressing transgenic plants is also rapid following transfer after DCE selection.

While comparable, the use of *dhlA* as a negative marker may have three advantages over the use of alcohol dehydrogenase (ADH). First, negative selection on allyl alcohol of plants expressing an ADH transgene requires the use of an ADH null allele background for transformation (López-Juez *et al.*, 1998). This limitation may complicate subsequent genetic analyses such as mapping studies. Because most plants do not express detectable haloalkane dehalogenase activity, the action of a *dhlA* transgene is not masked by an endogenous activity. As shown here, this should be true for a wide variety of plants other than *Arabidopsis*. Second, DCE is considerably less toxic than allyl alcohol, which may comfort users (Sax, 1984). However, both the aldehydes derived from DCE (2-chloroacetaldehyde) and allyl alcohol (acrolein) are highly reactive, their catabolites potentially mutagenic, and the accumulation of either may be equally toxic. Third, practical screening with *dhlA* does not require substrate selection in liquid culture, as may be the case for ADH (López-Juez *et al.*, 1998). It should be noted that, as for any marker-based, fusion genetic screen, the choice of a transgenic line expressing dhlA under the control of a promoter of interest, as well as the conditions for selection (substrate concentration and time course of incubation), must be empirically determined.

A question remains as to the exact mechanism of negative selection in plants with *dhlA*. By analogy to other systems, chloroacetaldehyde, derived from DCE via transgenic DhlA and endogenous ADH activities, is probably the major toxic catabolite produced (Janssen *et al.*, 1994). In keeping with this, we found that growth of *dhlA* transgenic plants is inhibited on chloroethanol (data not shown), perhaps due to the formation of chloroacetaldehyde via endogenous ADH. To directly address this question, we examined the growth in liquid culture of wild-type and ADH null plants in the absence and presence of 12 mM chloroethanol or allyl alcohol as a control (Dolferus *et al.*, 1990; López-Juez *et al.*, 1998). This concentration of substrates produced no detectable effects on plant growth after 2 days of incubation, in contrast to the toxic effect of the same concentration of DCE on transgenic dhlA plants (Figure 3). Continued incubation in chloroethanol for 14 days produced complete bleaching of wild-type, in contrast to ADH null plants which exhibited bleached leaves but green leaf bases, stems and meristem (data not shown). These symptoms are similar to those of dhlA transgenic plants treated briefly with DCE, as could be

seen microscopically prior to transfer in the reconstitution experiments (Figure 5).

This indicates that chloroacetaldehyde is the major toxic metabolite produced from DCE in dhlA transgenic plants, as suggested by the relative toxicities of DCE metabolites in animals (Sax, 1984). It also suggests that the effect of transgenic dhlA activity is cell autonomous, in that systemic cell death did not appear to occur in ADH null plants treated with chloroethanol or in dhlA transgenic plants under selection with DCE.

These results suggest that engineered expression of downstream aldehyde dehydrogenase (op den Camp and Kuhlemeier, 1997) and *dhlB* haloalkanoic acid dehalogenase, or of other detoxification pathways (Martinola *et al.*, 1993), might lead to the development of bioremediating plants capable of degrading DCE and related haloalkane pollutants. In any event, constitutive or regulated expression of *dhlA* may be used as a substrate-dependent negative marker in plants.

Experimental procedures

Construction of the dhlA *marker and analyses of transgenic plants*

The *dhlA* coding region was amplified from pKLA (Schanstra *et al.*, 1993) by PCR with *Vent* polymerase (New England Biolabs) using a 5′ *Xho*I linker-primer (*CTCGAG*CC**ATG**GTAAATG-CAATTCGCACC, start MET bold) and a 3′ *Xba*I linker-primer (*CTCAGA*CT**CTA**TTCTGTCTCGGCAAAGTG, stop bold). Following digestion with *Xho*I/*Xba*I, the fragment was directionally cloned in these sites as a transcriptional fusion in pKYLX71:35S2 (Schardl *et al.*, 1987). The plasmid was transferred by electroporation to *Agrobacterium* strain PGV3101 which was used to transform *Arabidopsis* ecotype Ws by vacuum infiltration (Bechtold *et al.*, 1993). Kan^R plantlets were selected by standard procedures and leaf DNA for PCR with *dhlA*-specific primers and total RNA for Northern analysis were isolated by mini-procedures (Klimyuk *et al.*, 1993; Nagy *et al.*, 1988). 10 μg total RNA per lane were separated in formaldehyde gels, blotted to Hybond N nylon filters and probed with *dhlA* coding sequences ^{32}P-labelled by nick translation after standard protocols.

Dehalogenase assays

Dehalogenase activity was determined by the release of halide in a colorimetric assay. 50 μl of extract was incubated with 3 ml of 5 mM substrate solution in 50 mM Tris buffer (pH 8.2) at 30°C. Halide production in 0.5 ml samples was determined spectrophotometrically at 460 nm with mercuric thiocyanate and ferric ammonium sulphate (Bergmann and Sanik, 1957). One activity unit was defined as the amount of enzyme that catalysed the formation of 1 μmol halide per minute.

The formation of the product 2-bromoethanol was analyzed by gas chromatography. A 0.5 ml sample was removed from the incubation mixture and extracted with 2 ml diethylether containing 0.05 mM 1-bromohexane as an internal standard. The diethylether fraction was injected on a Chrompack 483 gas chromotograph (Chrompack, Middelburg, the Netherlands) fitted with an electron capture detector. The separation of substrate and product was undertaken on a Chrompack CPWax 52 column with the following temperature program: 3 min isothermal at 60°C followed by an increase of 10°C/min to 240°C, and ending with an isothermal segment of 5 min at 240°C.

Negative selection with 1,2-dichloroethane

Seeds and mixtures of wild-type and transgenic seeds were surface sterilized and plated on MS media without or with 50 μg ml^{-1} kanamycin after autoclaving, or sown as a sand mix directly on 2–3 cm of autoclaved soil in a phytatray (Sigma). After cold treatment for 2 days at 4°C, plants were grown under 16 h light at 21°C. The effect of different DCE concentrations on the growth of wild-type and *dhlA* transgenics was initially examined after transfer of 10-day-old plants to liquid 0.5× MS for 4 days followed by 48 h treatment with DCE added to the media. For reconstitution experiments, volatile, liquid DCE in a microtube cap was placed in parafilm-sealed plates (5 μl) or phytatrays (30 μl) for selection of 10-day-old plants for 2 days under constant light. These liquid DCE volumes fully volatilize and correspond to approximately 1.5–2.0 mM DCE in air. Following selection on plates, plants were rescued by transfer to either MS or MS/KAN plates and fully recovered after 6–8 days of growth.

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Plant Physiol. (1994) 104: 1067–1071

Rapid Communication

The Herpes Simplex Virus Thymidine Kinase Gene as a Conditional Negative-Selection Marker Gene in *Arabidopsis thaliana*[1]

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The human herpes simplex virus thymidine kinase type 1 gene (*HSVtk*) acts as a conditional lethal marker in mammalian cells. The *HSVtk*-encoded enzyme is able to phosphorylate certain nucleoside analogs (e.g. ganciclovir, an antiherpetic drug), thus converting them to toxic DNA replication inhibitors. The utility of *HSVtk* as a conditional negative-selection marker was explored in *Arabidopsis thaliana* (L.) Heynh. *HSVtk* was introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation. Transgenic plants were morphologically indistinguishable from wild type and exhibited normal fertility. Ganciclovir at 10^{-5} to 10^{-4} M drastically reduced shoot regeneration on transgenic, HSVtk$^+$ root explants or callus formation on HSVtk$^+$ leaf explants but did not affect the wild-type cultures. There was a 35-fold reduction in shoot regeneration 8 d after transfer to shoot-induction medium. Negative selection against *HSVtk* activity along with kanamycin selection was also efficient in *Agrobacterium*-mediated gene transfer experiments. Shoot regeneration was 25 times lower on double-selective (ganciclovir plus kanamycin) plates than in the kanamycin control. This regeneration rate in double-selective plates is in the range of the frequency of shoots normally escaping kanamycin selection in *Arabidopsis* cultures.

Negative-selection marker genes are in increasing demand for genetic approaches toward understanding biological processes and for selecting cell types with desired alterations. The concept of negative selection is based on the expression of a marker gene that causes immediate or conditional cell lethality. It can be used when a particular class of cells needs to be eliminated. These markers may also be part of positive-negative-selection schemes for homologous recombination-mediated gene targeting (Capecchi, 1989). A potent negative-selection gene controlled by a promoter active during and after the transformation process will kill cells that have randomly integrated the vector and enrich for cells with the targeted mutation (or gene).

Expression of such suicide genes in particular cells of an organism may result in dominant "missing pattern" mutations (as discussed by Koncz et al., 1992) that may have special agronomic importance (i.e. male sterility). Suicide genes can be used to identify the place and time of promoter activity (Czakó et al., 1992) and mutants defective in signaling processes (Karlin-Neumann et al., 1992). Several nonconditional negative-selection markers are available for plant studies, such as the RNase T1 gene of *Aspergillus* (Mariani et al., 1990), the barnase gene from *Bacillus amyloliquefaciens* (Prior et al., 1991), the yeast *RAS2* gene (Hilson et al., 1990), the diphtheria toxin A chain (Koltunow et al., 1990; Mariani et al., 1990; Czakó and An, 1991; Thorsness et al., 1991; Czakó et al., 1992), the *Pseudomonas* gene encoding exotoxin A (Koning et al., 1992), and the anti-*nptII* gene (Xiang and Guerra, 1993).

Conditional lethal genes offer an added element of control over the negative selection through their dependency on externally provided substrates (lethal synthesis). The *tms2* gene of *Agrobacterium tumefaciens* T-DNA has been proposed as a tool for fundamental studies of gene inactivation (Depicker et al., 1988) and for identification of mutations in signaling processes in plants (Karlin-Neumann et al., 1992). The analogous *aux2* gene of the *Agrobacterium rhizogenes* T-DNA also has the potential as a negative-selection gene in *Brassica* at the plant level (Béclin et al., 1993).

Additional negative-selection genes used in other eukaryotic systems are considered for plant systems to develop one that can be used during the plant regeneration step after *Agrobacterium*-mediated gene transfer. The human herpes simplex virus thymidine kinase enzyme is able to phosphorylate certain nucleoside analogs that are not accepted by the cells' own kinase(s). For example, GAN, an antiviral drug, is converted to a toxic nucleotide analog that blocks DNA replication in mammalian cells (St. Clair et al., 1984). Thus, *HSVtk* acts as a conditional lethal marker, which has proven extremely useful in the enrichment for homologous recombinants in the mammalian system (Mansour et al., 1988).

Here we report the development of conditions for efficient negative selection with the herpes simplex virus thymidine kinase type 1 gene in *Arabidopsis thaliana*.

[1] Supported by U.S. Department of Energy grant No. DE-FG02–92ER20073.

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Abbreviations: GAN, ganciclovir or 9-(1,3-dihydroxy-2-propoxymethyl)guanine; *HSVtk*, herpes simplex virus thymidine kinase type 1 gene; KAN, kanamycin; MS, Murashige-Skoog; NAA, 1-naphthaleneacetic acid; *nptII*, neomycin phosphotransferase gene.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of wild-type *Arabidopsis thaliana* (L.) Heynh. genotype *RLD* (Langridge and Griffing, 1959) were originally provided by Dr. C.R. Somerville (Michigan State University, East Lansing). Since then, they have been harvested in a growth chamber at 24°C under 16 h of illumination with a mixture of fluorescent and incandescent light. Irradiance, 80 μmol m^{-2} s^{-1}, and 70% RH were measured by a steady-state porometer (LI-1600; Li-Cor, Inc., Lincoln, NE). Media for seed germination, root pretreatment (ARMI), and shoot induction (ARMIIr) were the same as used by Márton and Browse (1991), except the regenerated plantlets were directly transferred to MS medium containing 0.2 mg L^{-1} of NAA and no vitamins, omitting the shoot multiplication step on ARMIII. The phytohormone composition for ARMI was 3 mg L^{-1} of IAA, 0.15 mg L^{-1} of 2,4-D, 0.6 mg L^{-1} of BA, and 0.3 mg L^{-1} of isopentenyl adenine; for ARMIIr the phytohormone composition was 0.2 mg L^{-1} of NAA and 4 mg L^{-1} of isopentenyl adenine. All phytohormones were filter sterilized and added to precooled (45°C) media.

Transgenic *A. thaliana* plants were obtained by the root transformation protocol of Márton and Browse (1991), except that acetosyringone was not used in the bacterial culture medium and the subculture on ARMIII was omitted (see above). About 40 cm of roots were cultured in a plate. Putative transformants were selected on ARMIIr containing 50 mg L^{-1} KAN. A commercial formulation (Timentin; SmithKline Beecham Pharmaceuticals, Philadelphia, PA) of the penicillin ticarcillin and the β-lactamase inhibitor clavulanic acid was incorporated in the medium at 400 mg L^{-1} to eliminate *Agrobacterium*. Resistance to KAN was verified by rooting shoots on MS medium containing 0.2 mg L^{-1} of NAA. MS medium is MS salts (Murashige and Skoog, 1962), 3% (w/v) Suc, and 3 mL L^{-1} of Miller's solution (6% [w/v] KH_2PO_4). KAN-resistant leaf segments were able to form callus on 50 mg L^{-1} of KAN-containing C medium (similar to MS, except for 1.0 mg L^{-1} of NAA, 100 mg L^{-1} of *myo*-inositol, and 1 mg L^{-1} of thiamine hydrochloride). Individual transgenic plants were vegetatively propagated as root cultures (Czakó et al., 1993).

GAN-free base or its sodium salt (as Cytovene; Syntex Laboratories, Palo Alto, CA) was dissolved in water, filter sterilized, and added to the autoclaved media. GAN sensitivity of transgenic plants was tested on solid RMOP medium (same as C except for 1.0 mg L^{-1} of BA and 0.1 mg L^{-1} of NAA [Maliga, 1984]).

Bacterial Strains and Plasmids Used

The pGA643 (An et al., 1988), pGA972 (Czakó et al., 1992), and pCX305.1 (Fig. 1; M. Czakó, R.P. Marathe, C. Xiang, D.J. Guerra, G.J. Bishop, A. Jones, L. Márton, unpublished data) binary vectors were transferred into *Agrobacterium tumefaciens* strains LBA4404 (Hoekema et al., 1983) and EHA105 (Hood et al., 1993), both carrying a disarmed helper Ti plasmid, by the direct transformation method (An, 1987). *Agrobacterium* strains were grown on YEP solid medium or in liquid AB minimal medium (An, 1987) in the presence of appropriate antibiotics: 3 mg L^{-1} of tetracycline, 7.5 mg L^{-1} of KAN, 200 mg L^{-1} of streptomycin, and 25 mg L^{-1} of rifampicin.

Determination of Bacterial Counts and GAN Sensitivity

The inhibitory effect of GAN was tested by adding 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M GAN into wells made in YEP nutrient agar plate (1.5% agar) that had been inoculated by overlaying with the *Agrobacterium* strain resuspended (100 μL of an overnight suspension diluted to an A_{600} of 1.0) in soft agar (0.7%) YEP medium. The plates were examined for inhibition zones representing minimal inhibitory concentrations after 24 h.

To determine viable bacterial cell density in different stages of co-cultivation and selection, samples were collected from the liquid medium and from the roots. From the root samples bacteria were liberated by squashing with a glass rod in a conical tube in liquid YEP medium. Colony-forming units were determined by plating aliquots from serially diluted samples on YEP medium.

Genomic DNA Analysis

Total DNA was prepared from leaf tissue by the cetyltrimethyl-ammonium bromide method (Rogers and Bendich, 1988). DNA samples digested with *Hin*dIII or *Kpn*I were fractionated on a 0.7% (w/v) agarose gel in 0.5 × Tris-borate-EDTA buffer and then transferred onto a Zeta-Probe membrane (Bio-Rad) by alkaline blotting using the manufacturer's protocol. Probe DNA was labeled with ^{32}P using the random priming procedure of the manufacturer (Amersham) and hybridized to the membrane according to standard procedures (Sambrook et al., 1989).

RESULTS AND DISCUSSION

Callus and Shoot Formation Is Inhibited by GAN on Explants of Herpes Simplex Virus Thymidine Kinase Transformants

The feasibility of *HSVtk* as a conditional negative-selection marker was explored in a plant system. *HSVtk* was fused to a cauliflower mosaic virus 35S RNA promoter derivative that is present on a binary vector carrying a plant-selectable KAN-resistant *nptII* (Fig. 1). The chimeric construct was stably transformed into *A. thaliana* by *Agrobacterium*-mediated gene transfer. KAN-resistant plants were subjected to genomic DNA gel blot analysis with probes covering *HSVtk* or *nptII*. The *HSVtk* probe detected the *HSVtk* sequences in most KAN-resistant transformants (Fig. 2, lane 2).

Transgenic plants harboring the *HSVtk* construct ($HSVtk^+$) were phenotypically indistinguishable from wild-type plants and exhibited normal morphology and fertility. Conditions were sought under which the plant material carrying *HSVtk* exhibited a distinct phenotype upon addition of the toxigenic substrate, GAN. GAN sensitivity was not manifested at the seedling level. Self-progeny of a randomly picked $HSVtk^+$ (RLD305–1, Fig. 2, lane 2) plant and wild-type seeds were germinated in the presence of 10^{-4} M GAN, 50 mg L^{-1} KAN, or both. Seedlings of the RLD305–1 transgenic plant were

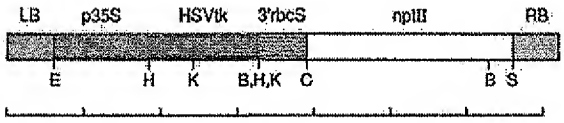

Figure 1. Physical organization of T-DNA of the *A. tumefaciens* pCX305.1 binary vector used for introducing *HSVtk* into *Arabidopsis*. *HSVtk* (from nucleotide positions 263 to 1799; McKnight, 1980; Mansour et al., 1988) is expressed from a cauliflower mosaic virus 35S RNA promoter (p35S) and terminated by the *rbcS*-E9 polyadenylation region (3'rbcS; Hunt and McDonald, 1989) in a pKYLX7-type (Schardl et al., 1987) binary plant expression vector, pKYLX7S2. The 35S RNA promoter in pKYLX7S2 (Dr. A.G. Hunt, University of Kentucky, Lexington, personal communication) is enhanced by a duplication of the region that extends from −417 to −90 with respect to the 35S RNA transcription start site (Guilley et al., 1982). The *nptII* chimeric KAN-resistance gene is under the control of the *Agrobacterium* nopaline synthase promoter and terminator regions (An, 1987). Abbreviations for restriction enzymes: B, BamHI; C, ClaI; E, EcoRI; H, HindIII; K, KpnI. Scale is in kb.

indistinguishable from wild-type seedlings on GAN-containing medium. Segregation for KAN resistance (approximately 3:1) was not affected either. Explant growth assays were also used to test whether GAN had any adverse effects on normal tissues and to determine whether the HSVtk trait was manifested in the transgenic plants. Callus growth assay was carried out on wild-type plants and a randomly picked HSVtk$^+$ (RLD305–1) plant on GAN-containing medium. Leaf segments were incubated on callus-inducing (RMOP) medium containing GAN at concentrations from 10^{-7} to 10^{-4} M. GAN (10^{-4} M) proved to be completely inhibitory to callus initiation on HSVtk$^+$ leaf segments, whereas callus formation on wild-type leaf segments was not affected (Table I).

These observations prove that the GAN-*HSVtk* gene system

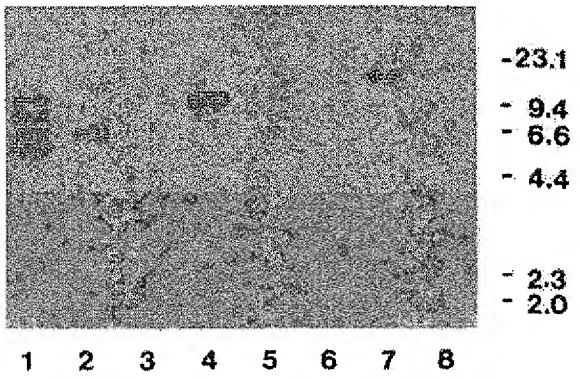

Figure 2. Detection of *HSVtk* sequences in *Arabidopsis* plants transformed by pCX305.1. Southern blots of EcoRI-digested DNA samples were probed with the 1.58-kb HindIII fragment covering *HSVtk* 5(Fig. 1). Lane 1, RLDTK1–9 (Kan^R); lane 2, RLD305–1 (Kan^R); lane 3, RLDTKG14/15–2 (KAN^S); lane 4, RLDTKG6/7–1 (Kan^R); lane 5, RLDTKG4–4 (KAN^S); lane 6, RLDTKG2–1 (KAN^S); lane 7, RLDTKG4–1 (Kan^R); lane 8, *RLD* wild type. Sizes (kb) of phage λ-DNA HindIII fragments, separated on the same gel, are shown on the right.

Table I. *Effect of GAN on callus induction on transgenic Arabidopsis leaf explants*

GAN Concentration	Callus Wt Gain	
	HSVtk$^+$	HSVtk$^-$
M	% of control	
0	100	100
10^{-7}	94	96
10^{-6}	74	85
10^{-5}	22	85
10^{-4}	0	108

fulfills the following conditions for utility as a conditional negative-selection marker: (a) the substrate used in "lethal synthesis" should not be toxic to normal plant cells, (b) there should be an absence of any intrinsic enzyme activity capable of converting the substrate into a toxic metabolite in plants, and (c) the reaction carried out by the transgene should be sufficiently specific to minimize interference with normal cellular metabolism.

Sensitivity of the shoot-regeneration process from root segments to 10^{-4} M was tested, because this is the procedure of choice for mass transformation of *Arabidopsis*. To simulate transformation conditions, KAN was also included in the media, and a KAN-resistant HSVtk$^-$ plant (line RLD972–1; transformed by pGA972 carrying the same KAN-resistance gene but without *HSVtk*) was also used as a control.

Explants taken from amplified root cultures (Czakó et al., 1993) of HSVtk$^+$ (line RLD305–1), HSVtk$^-$ (line RLD972–1), and wild-type *RLD* plants (line RLD-1, Czakó et al., 1993) were induced to regenerate shoots by a two-step protocol (Márton and Browse, 1991). Either 10^{-4} M GAN, 50 mg L^{-1} of KAN, both, or neither was included in ARMIIr medium during shoot induction. GAN drastically reduced shoot regeneration from HSVtk$^+$ root explants.

The difference between the wild-type and HSVtk$^+$ cultures was particularly striking at the earliest stages of shoot regeneration (8 d after transfer to the ARMIIr medium), with 371 versus 11 dark green foci per plate (not shown). The few shoots that eventually developed on the HSVtk$^+$ roots appeared to be normal. Since no rearrangement could be detected in *HSVtk* as judged from DNA gel blot hybridizations with an *HSVtk*-specific probe, it may be assumed that these plants escaped negative selection by silencing the foreign DNA, e.g. by methylation (Renckens et al., 1992).

From the observations that GAN inhibited callus initiation on leaf segments and shoot regeneration on transgenic leaf segments but not the growth of established transgenic callus (not shown), it appears that the initiation step is sensitive to GAN. Because of the large nucleotide pools observed in cultured callus cells (Márton et al., 1978), the nucleotide analog GAN is probably more efficiently removed from the DNA or not even incorporated frequently enough to cause inhibition of replication.

HSVtk as a Negative Selectable Marker in *Arabidopsis*

Direct shoot regeneration from root explants is a frequently used method for the generation of transgenic *Arabidopsis*,

because the resulting transgenic shoots can be immediately subjected to genetic analysis. *HSVtk* was, therefore, tested in model transformation experiments under a positive-negative-selection regime using conditions established on HSVtk$^+$ transgenic lines.

Arabidopsis root explants were co-cultivated with *Agrobacterium* carrying either pCX305.1 (HSVtk$^+$) or pGA643 (HSVtk$^-$, empty vector) plasmids or with neither plasmid. GAN selection (10^{-4} M) was started 3 d after inoculation, at the shoot-induction stage on ARMIIr medium, simultaneously with KAN selection (50 mg L^{-1}). By this time the gene transfer from *Agrobacterium* to the plant cells is considered to have already taken place.

GAN reduced shoot regeneration from root explants cultured on KAN-containing medium after transformation with pCX305.1 (Fig. 3), where the *HSVtk* and the KAN-resistance markers are linked on the T-DNA. The inhibitory effect was as striking at the earliest stages of shoot differentiation as in the model experiments with established transgenic root cultures: 2 versus 50 KAN-resistant shoots (>3 leaves) were observed per five plates. A 4-fold difference in the number of shoots remained even after 4 weeks on shoot-regeneration medium (not shown), at which time shoots developed from KAN-sensitive cells that escaped selection (Márton and Browse, 1991). The low number of regenerating shoots on the double-selection (i.e. GAN and KAN) plates indicates efficient negative selection against HSVtk$^+$ transformants. This inhibition is consistent with the effect on root explants of a pure HSVtk$^+$ transgenic line (RLD305–1). In the case of the empty-vector (pGA643) plasmid, the regeneration frequency was not affected (85 versus 85 shoots).

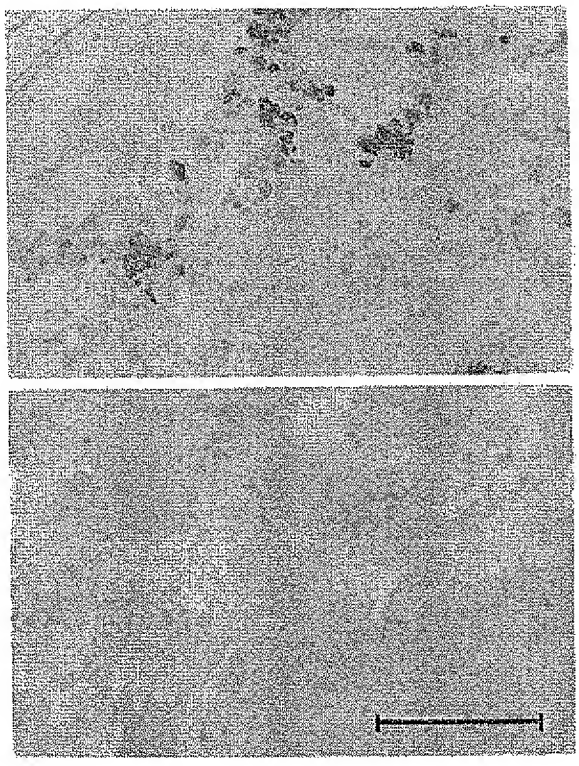

Figure 3. GAN inhibits regeneration of shoots on *Arabidopsis* root explants transformed with *Agrobacterium* carrying the HSVtk$^+$ binary vector. Top, Strain EHA105(pCX305.1). Bottom, EHA105 binary vector-free control strain. Twelve days after transfer to the ARMIIr shoot-induction medium containing 10^{-4} M GAN and 50 mg L^{-1} of KAN. Bar represents 1 cm.

An interference of GAN with the transforming bacteria is not likely. By the time GAN selection started, the majority of bacterial cells were removed by rinsing the roots at the end of the 2-d co-cultivation, and the remaining cells were subjected to the presence of an antibiotic. In addition, the *Agrobacterium* strains with or without the HSVtk$^+$ plasmid (pCX305.1) were not inhibited by GAN even at 10^{-4} M in a standard agar diffusion assay. Nor was the residual *Agrobacterium* titer reduced by GAN in the presence of root tissues (i.e. after 5 d on ARMIIr medium) in the co-cultivation experiment. These results suggested that background from *HSVtk* expression by the cauliflower mosaic virus 35S RNA promoter (Janssen and Gardner, 1989) of pCX305.1 would have negligible effect on the bacteria.

Typical for a population of plants collected in a leaf-segment transformation experiment, DNA-DNA hybridization with radioactively labeled probes revealed different marker combinations: *nptII/HSVtk*-positive lines, *nptII*-positive lines, and nontransformed lines. A 1.58-kb *Hin*dIII probe (Fig. 1) containing *HSVtk* hybridized with up to four bands in *Eco*RI-digested DNA isolated from transgenic plants, whereas DNA from the wild-type plant (Fig. 2, lane 8) and from KAN-sensitive plants that escaped selection showed no hybridization signal (lanes 3, 5, and 6). The *HSVtk* probe also detected the 1.0-kb internal fragment of the *HSVtk* gene (Fig. 1) as well as up to three border junction fragments with *Kpn*I-digested DNA. On the same blot the *nptII*-specific probe (2.1-kb *Cla*I-*Bam*HI fragment) showed hybridization only to samples from KAN-resistant plants corresponding to lanes 1, 2, 4, and 7 (not shown).

The experiments presented here demonstrate that the herpes simplex virus thymidine kinase gene fulfills the requirements for a conditional negative-selection marker in *Arabidopsis*. With appropriate culture conditions and optimal timing, the inhibitory effect of GAN on transgenic HSVtk$^+$ plants may translate into a positive-negative-selection regime (Mansour et al., 1988) that could enrich for homologous recombination events in gene-targeting experiments. A positive-negative-selection-targeting vector would contain a region of DNA homologous to a gene, interrupted within an exon by the *nptII*-positive-selection element, with the *HSVtk*-negative-selection element ligated onto the end. This marker arrangement is designed to enrich for targeted clones, since cells with a randomly integrated targeting vector have a high probability of retaining both positive and negative-selection elements, whereas replacement-type homologous recombination at the targeted locus often excludes the negative-selection element. Double selection for the presence of *nptII* and the absence of *HSVtk* (by culture in the presence of KAN and GAN) would result in a reduced number of stably transfected clones with a randomly inserted positive-negative-selection vector and a concomitant enrichment for homologously recombined clones. Unlike with the nonconditional negative-selection markers, the time and level of negative selection can be controlled. From these experiments

negative selection against transgenic *Arabidopsis* shoots carrying an intact *HSVtk* may reduce (up to 25-fold) the number of transformed plants one has to deal with in a gene-targeting experiment.

GAN toxicity was observed also with HSVtk$^+$ transgenic tobacco, but there was considerable variability in GAN sensitivity between individual transformants (M. Czakó, R.P. Marathe, C. Xiang, D.J. Guerra, G.J. Bishop, A. Jones, L. Márton, unpublished data).

During the revision of this manuscript, it came to our attention that the *Escherichia coli* cytosine deaminase gene (*codA*) was also shown to provide substrate-dependent toxicity in tobacco, *Lotus japonicus* (Stougaard, 1993), and *Arabidopsis* (Perera et al., 1993). However, negative selection at the level of transformation has not been reported with this marker.

ACKNOWLEDGMENTS

We are grateful to Dr. John Fried, Syntex Research Inc., for a gift sample of GAN and technical information, Dr. Daniel J. Guerra for providing the pCX305.1 plasmid, and Dr. Arthur G. Hunt for providing information concerning the pKYLX7S2 plasmid. We thank Allan R. Wenck for critical reading of the manuscript.

Received September 27, 1993; accepted December 3, 1993.

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The methionine salvage pathway in *Bacillus subtilis*

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Published: 25 April 2002

BMC Microbiology 2002, **2**:8

Received: 4 March 2002

Accepted: 25 April 2002

This article is available from: http://www.biomedcentral.com/1471-2180/2/8

Abstract

Background: Polyamine synthesis produces methylthioadenosine, which has to be disposed of. The cell recycles it into methionine through methylthioribose (MTR). Very little was known about MTR recycling for methionine salvage in *Bacillus subtilis*.

Results: Using *in silico* genome analysis and transposon mutagenesis in *B. subtilis* we have experimentally uncovered the major steps of the dioxygen-dependent methionine salvage pathway, which, although similar to that found in *Klebsiella pneumoniae*, recruited for its implementation some entirely different proteins. The promoters of the genes have been identified by primer extension, and gene expression was analyzed by Northern blotting and *lacZ* reporter gene expression. Among the most remarkable discoveries in this pathway is the role of an analog of ribulose diphosphate carboxylase (Rubisco, the plant enzyme used in the Calvin cycle which recovers carbon dioxide from the atmosphere) as a major step in MTR recycling.

Conclusions: A complete methionine salvage pathway exists in *B. subtilis*. This pathway is chemically similar to that in *K. pneumoniae*, but recruited different proteins to this purpose. In particular, a paralogue or Rubisco, MtnW, is used at one of the steps in the pathway. A major observation is that in the absence of MtnW, MTR becomes extremely toxic to the cell, opening an unexpected target for new antimicrobial drugs. In addition to methionine salvage, this pathway protects *B. subtilis* against dioxygen produced by its natural biotope, the surface of leaves (phylloplane).

Background

The fate of methylthioribose (MTR), the end-product of spermidine and spermine metabolism, as well as of ethylene biosynthesis has not yet been fully explored in most organisms. In *Escherichia coli* this molecule is excreted in the medium [1] while in *Klebsiella pneumoniae* it constitutes the methionine salvage pathway, being metabolized back into methionine [2,3]. In eukaryotic parasites it is also recycled into methionine, presumably through a pathway similar to that in *K. pneumoniae*[4]. In *Bacillus subtilis* we found that MTR is an excellent sulfur source [5] and we unraveled some of the steps involved in its metabolism, which starts from phosphorylation of MTR, mediated by the MtnK protein [6].

It has been shown previously that the *ykrW* gene, annotated as similar to ribulose phosphate carboxylase/oxygenases (Rubisco) in the original sequence, has links with sulfur metabolism. Indeed, Henkin and co-workers found that the corresponding coding sequence (CDS) was preceded

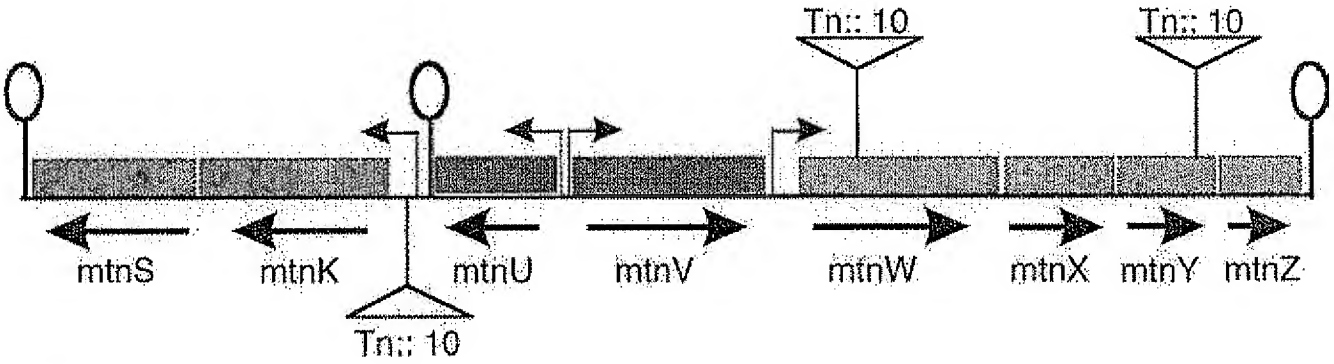


Figure 1
Location of transposon (Tn*10*) insertions in the *mtn* region. One insertion was localized 73 bp upstream of the translational start point of the *mtnK* gene [6], four were located into *mtnW* and six into the *mtnY* gene. The insertion situated 353 bp downstream of the *mtnW* translation start point (strain BSHP7064) and one situated 556 bp downstream of the *mtnY* translation start point (strain BSHP7065) are shown in the figure.

by a S-box typical of sulfur metabolism genes in *B. subtilis*[7] and Hanson and Tabita found that two classes of enzymes similar to Rubisco were associated with sulfur metabolism [8]. Interestingly, these authors, working with *Chlorobium*, which uses the reverse TCA cycle for CO_2 fixation, postulated these proteins as possible precursors of cyanobacteria, then plant Rubisco at an early time of the development of life on Earth.

This raised interesting questions about the origin of this pathway. In particular the YkrW gene origin could have been early in evolution, or resulting from lateral transfer from plants to bacilli. We demonstrate here that proteins YkrUWXYZ are needed for MTR recycling into methionine in *B. subtilis*, while YkrV, an aminotransferase, is probably more specific of methionine transamination, but is dispensable in the present conditions because of the present of a variety of izozymes (up to nine amino acid transaminases are present in *B. subtilis*).

Results

Transposon insertion mutations and phenotype of inactivated mutants

The MTR analog trifluormethylthioribose (3F-MTR) is toxic if the methyl sulfur moiety of the molecule is recycled [9]. This molecule was therefore an excellent candidate to explore the steps needed for MTR recycling to methionine. Mutants were obtained by transformation of a wild type strain with a random transposon library, selecting for growth in the presence of 3F-MTR in the presence of sulfate as sulfur source. The mutants were subsequently tested for growth on plates lacking sulfur source but supplemented with MTR: only those that could not grow were retained for further study. In order to ascertain that the resistant phenotype was not coming from secondary mutations but was directly related to the transposon insert, the chromosome DNA was extracted from each putative mutant and back transformed into a wild type strain selecting for the transposon antibiotic marker. The 3F-MTR and MTR phenotypes were subsequently tested and only those mutants that passed the test were retained. The insertion positions of the transposons were then sequenced. As shown in Figure 1 we recovered mutants in several genes located in the close vicinity of each other. One mutant was located at the *mtnK* locus (previously named *ykrT*[6]), four were located into *ykrW* and six into the *ykrY* gene. One clone with transposon insertion into the *ykrW* gene (strain BSHP7064, insertion situated 353 bp downstream of *ykrW* translation start point) and one into the *ykrY* gene (strain BSHP7065, insertion situated 556 bp downstream of *ykrY* translation start point) were retained for further studies.

Using the collection of the *lacZ* transcriptional fusion mutants constructed during the *Bacilllus subtilis* functional analysis program ([http://locus.jouy.inra.fr/cgi-bin/genmic/madbase/progs/madbase.operl] and [http://bacillus.genome.ad.jp] , [10]) and constructing mutants which were not available in the collection, we tested all genes in the region for their phenotype of growth on MTR as the sole sulfur source. Table 1 displays the results obtained. As we can see, mutants in *mtnK* (previously identified as coding for MTR kinase [6], strain BFS1850), *mtnS* (strain BSHP7010 [6]), *ykrU* (renamed *mtnU*, strain BFS1851), *ykrW* (renamed *mtnW*, strain BSHP7014 allowing the expression of downstream genes) and *ykrY* (renamed *mtnY*, strain BSHP7016) failed to grow on the substrate. In the absence of IPTG *ykrX* (renamed *mtnX*, strain BFS1852) also failed to grow, but it recovered its growth properties when IPTG was added to the medium, suggesting some polar effect of the transposon insertion. The mutant of *ykrZ* (renamed *mtnZ*, strain BFS1853) presented only a

Table 1: Phenotype of gene inactivation in the *mtn* region.

Gene name	Strain	Growth on MTR as sole sulfur source
Wild type +O2[a]	168	normal growth after four days
Wild type -O2[a]	168	no growth after four days
mtnS (*ykrS*)[b]	BSHP7010	no growth
mtnK (*ykrT*)	BFS1850	no growth
mtnU (*ykrU*)	BFS1851	no growth (numerous revertants)
mtnV (*ykrV*)	BSHP7020	normal growth
mtnW (*ykrW*)	BSHP7014	no growth
mtnX (*ykrX*)	BFS1852	normal growth
mtnY (*ykrY*)	BSHP7016	no growth
mtnZ (*ykrZ*)	BFS1853	weak residual growth

a. See Materials and methods; nitrate was used as an electron acceptor. **b.** Former gene names are given in brackets.

very weak (residual) growth on MTR, suggesting the presence in the cell of some other enzymatic activity able to partially complement the lack of *mtnZ* gene product. Disruption of *ykrV* (renamed *mtnV*, strain BSHP7020) had no visible effect on growth on MTR as the sulfur source.

Identification of promoters

Several genes in the region have been shown by Henkin and co-workers to be expressed from promoters regulated by the S-box attenuation system [7]. This is the case of *mtnKS* and *mtnWXYZ* transcription units. Some of the genes, however, are not regulated in this way. Expression of the *mtnU* and *mtnV* genes is not subject to that regulation since no S-box is present in their leader transcript. As shown in Figure 2A the promoter of *mtnU* is located 35 nt from the translation start point. Its start was found to lie 5 nt downstream from a putative -10 box identified in the sequence (TTAAAT). Upstream from this box separated by 18 nt is a -35 box (ATGATA) with sequence similar to the consensus sequence TTGACA that is typical of *B. subtilis* sigmaA-dependent promoters [11].

The promoter of *mtnV* is located 42 nt upstream from the translation start point. Its start lies 8 nt downstream from a putative -10 box identified in the sequence (TATGAT) separated by 17 nt from -35 box (TTTACT) (see Fig. 2.B). The *mtnU* and *mtnV* genes share the same promoter region (94 nt) but are transcribed in divergent orientation from overlapping promoters. Thus, the -10 box of the *mtnV* promoter is situated between the -10 and -35 boxes of the *mtnU* promoter and the -10 box of the *mtnU* promoter is situated between the -10 and -35 boxes of the *mtnV* promoter.

The *mtnKS* promoter region is 326 nt long. Its start was found to lie 7 nt downstream from a putative -10 box identified in the sequence (TACCAT) (see Fig. 2.C). Upstream from this box and separated by 18 nt is a -35 box (TTGACA), a typical *B. subtilis* sigmaA-dependent promoter. Downstream of this promoter lies an S-box regulatory sequence.

Genes *mntWXYZ* are expressed from two overlapping promoters that are situated in a 195 nt long region. The upstream P1 promoter's start was found to lie 7 nt downstream from a putative -10 box identified in the sequence (GATAAT) separated by 17 nt from a consensus -35 box (TTGACA). The P2 promoter's start was found to lie 7 nt downstream from a putative -10 box identified in the sequence (TAAAAT) upstream from which is a -35 box (ATGGGA) (see Fig. 2.D). This promoter region is also reminiscent of regions recognized by the developmental sigmaH transcriptional regulator. The -10 box of the P1's promoter and the -35 box of P2 are partly overlapping. The relative intensity of the signals indicates that transcription from the P1 promoter is more abundant than from P2 (see Fig. 2.D).

Transcription organisation of the mtn locus

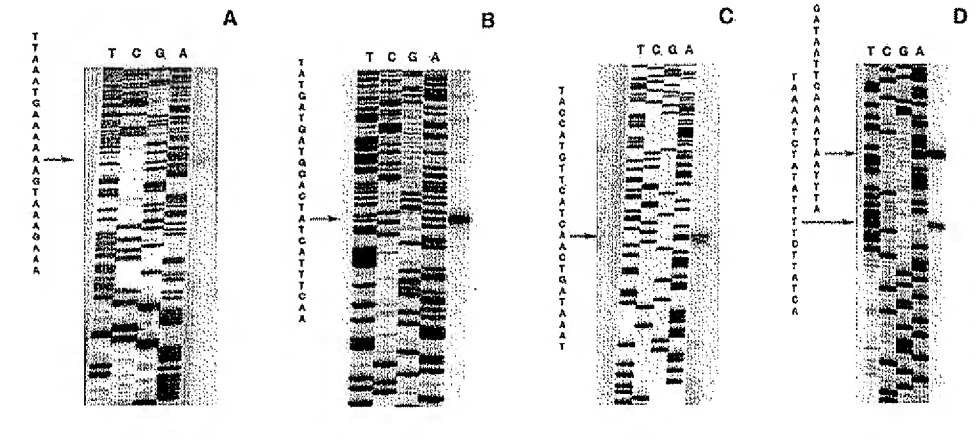
To further investigate transcription of the *mtnVWXYZ* genes, RNA synthesis was analyzed by Northern blotting. RNA was extracted from exponentially growing cells, in minimal medium containing either sulfate or methionine as sulfur source. As shown in Figure 3.A, a band of about 1200 nt, corresponding to the expected length of a transcript initiated at the *mtnV* promoter and terminating near its stop codon, was observed for the *mtnV* gene probe. An equal intensity of the signal was observed for *mtnV* transcripts prepared from cells either grown with sulfate or with methionine as sulfur source (lanes 1 and 2, Fig. 3.A).

When *mtnW* and *mtnZ* gene specific probes were used, two bands were revealed: one of about 2.5 kb and second of about 3.2 kb (Fig. 3.B and 3.C)). The larger band corresponds to the expected length of a transcript initiated at the *mtnW* promoter and terminating in a stem and loop structure at the end of the *mtnWXYZ* transcriptional unit. The smaller band can possibly be the result of RNA processing at the end of the S-box regulatory sequence of the 5' extremity of the transcript. The intensity of bands when hybridizing RNA from cells grown with sulfate as sulfur source was higher than when using RNA from cells grown in the presence of methionine (lane 1 and 2 in Fig. 3.B and 3.C).

As shown previously, *mtnK* and *mtnS* are expressed as an operon, while *mtnU* is expressed independently [6].

Regulatory features

To substantiate the results obtained with RNA analysis and further investigate the expression of genes from the



E

mtnU: tttcatctattctcaccttctaaaaaaatcttgaaATGATAgtccatcatcatatatggTTAAATg
-35 -10
aaaaaagtaaagaaaaatttggggtgttaaaaag ATG AAA TGG - - -
+1 mRNA

mtnV: catctttttaacaccccaaatttttcTTTACTtttttcatttaaccataTATGATgatggactat
-35 -10 +1 mRNA
catttcaagatttttttagaaggtgagaatag ATG AAA TTT - - -

mtnKS: aaaaaatataTTGACAactaactaaattacctgtTACCATgttcatcaactgataaattagaat
-35 -10 +1 mRNA
tgtcaaacaagtgaatatattctcttatcgagagttgggcgagggatttggccttttgacccca
aaagcaaccgaccgtaattccattgtgaaatggggcgcatttttttcgcgccgagacgctggtc
tcttaaggcacggtgctaattccattcagatctgatctgagagataagagaggcggacatagat
gttaagcctccttctctctgagaaggaggctttttttacggccacatattaattaattacataat
tggaggttatg ATG ATG ATG - - -

mtnW: caaactgaacctgtttacccaaaaaagcaTTGACAagaccttataacgATGGGATAATTcaaaat
-35 -35 -10
aatttaTAAAATctatatttcttatcaagagcaggcagagggacgagcccgatgaagcccggca
+1 mRNA -10 +1 mRNA
accgacttataaagcacggtgctaattcttgcagctagcggctgagagataagattcggacgaga
aacgaaacctctttagacgcgattgcagtttgaagaggttttttgat ATG GAT GAA - - -

Figure 2
Identification of the *mtn* region promoters by primer extension. A. Identification of the transcription start site of the *mtnU* operon. The size of the extended product is compared to a DNA-sequencing ladder of the *mtnU* promoter region. Primer extension and sequencing reaction were performed with the same primer. The +1 site is marked by an arrow. B. Identification of the transcription start site of the *mtnV* gene. The size of the extended product is compared to a DNA-sequencing ladder of the *mtnV* promoter region. Primer extension and sequencing reaction were performed with the same primer. The +1 site is marked by an arrow. C. Identification of the transcription start site of the *mtnKS* gene. The size of the extended product is compared to a DNA-sequencing ladder of the *mtnKS* promoter region. Primer extension and sequencing reaction were performed with the same primer. The +1 site is marked by an arrow. D. Identification of the transcription start site of the *mtnWXYZ* operon. The size of the extended product is compared to a DNA-sequencing ladder of the *mtnWXYZ* promoter region. Primer extension and sequencing reaction were performed with the same primer. Two +1 sites are marked by arrows. E. Sequences of the corresponding promoter regions. Promoter sites are in capital letters and underlined (-35 and -10 boxes), and the transcription start sites are indicated by broken arrows (+1).

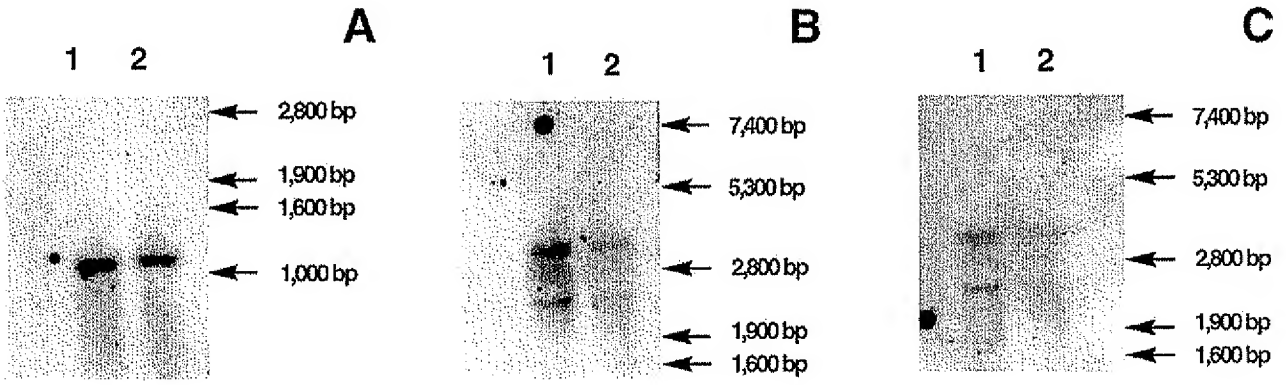


Figure 3
Northern blot analysis of *B. subtilis* 168 *mtnVWXYZ* region. A total of 3 μg of RNA was used. A. Northern hybridization with *mtnV* gene specific probe. RNA corresponding to lane 1 was obtained from a culture grown in minimal medium with sulfate as a sulfur source, and for lane 2 from a culture grown in minimal medium with methionine as a sulfur source. B. Northern hybrydization with *mtnW* gene specific probe. RNA corresponding to lane 1 was obtained from a culture grown in minimal medium with sulfate as a sulfur source, and for lane 2 from a culture grown in minimal medium with methionine as a sulfur source. C. Northern hybrydization with *mtnZ* gene specific probe. RNA corresponding to lane 1 was obtained from a culture grown in minimal medium with sulfate as a sulfur source, and for lane 2 from a culture grown in minimal medium with methionine as a sulfur source.

mtn region, we constructed mutants carring lacZ transcriptional fusions as well as used some mutants constructed during the functional analysis program (also corresponding to lacZ transcription fusions). Table 2 shows the results obtained with these strains when using sulfate or methionine as sulfur source. The mtnU gene (strain BFS1851, mtnU::lacZ) is expressed constitutively at a fairly low level and its expression is independent of the sulfur source used (62 U/mg of protein in the exponential growth phase in presence of sulfate and 53 U/mg of protein in the exponential growth phase in presence of methionine). In contrast, the mtnV gene (strain BSHP7020, mtnV::lacZ) although expressed in the similar way (constitutive and sulfur source independent expression) is expressed at a significantly higher level (217 U/mg of protein in the exponential growth phase in presence of sulfate and 181 U/mg of protein in the exponential growth phase in presence of methionine).

The genes from the *mtnWXYZ* transcriptional unit (strains BSHP7014, BFS1852, BSHP7016 and BFS1853 for *mtnW::lacZ*, *mtnX::lacZ*, *mtnY::lacZ* and *mtnZ::lacZ*, respectively) are expressed in a coordinated and sulfur source-dependent way. The expression of the first gene in the operon (*mtnW*) is higher than that of the last one (*mtnZ*) with intermediary values for intermediary genes *mtnX* and *mtnY*. This suggests the effect of some transcription polarity during the process of transcription (see Table 2). A 5-fold difference is observed between the expression of the *mtnWXYZ* genes in the presence of sulfate and that in the presence of methionine (579 U/mg of protein in the exponential growth phase in the presence of sulfate and 113 U/mg of protein in the exponential growth phase in the presence of methionine for the *mtnW::lacZ* transcriptional fusion and 280 U/mg of protein in the exponential growth phase in presence of sulfate and 57 U/mg of protein in the exponential growth phase in presence of methionine for *mtnZ::lacZ* transcriptional fusion). This observation is in accordance with the presence of S-box regulatory element in the promoter region of *mtnWXYZ* operon which modulates gene expression as a function of methionine availability [7].

Reconstruction of the metabolic pathway

In order to identify the methionine salvage pathway we made constructs allowing us to decipher the order of the gene products in the pathway, together with *in silico*, physiologic and genetic analysis of the effect of metabolites of the pathway. This is reminiscent of the way advocated by Koonin *et al.* for the use of *in silico* approaches as complement to *in vivo* experiments [12].

As a first goal we showed that the end product of the pathway is indeed methionine. This was demonstrated by showing that MTR, which is a good sulfur source, can be used as the methionine source in methionine auxotrophs (Fig. 4, Fig. 5 and data non shown).

Two genes in the pathway are dispensable, *mtnV* and *mtnX*. The first one encodes a transaminase of which there

Table 2: Expression of *mtn::lacZ* transcriptional fusions.

	β-galactosidase Activity (U mg-1 of protein)[a]			
	ED1 medium with sulfate		ED1 medium with methionine	
Strain	exp[b]	stat	exp	stat
BFS1851[c]	62	41	53	33
BSHP7020	217	121	181	161
BSHP7014	579	267	113	95
BFS1852	442	251	108	92
BSHP7016	294	139	61	47
BFS1853	280	112	57	33

a. for the β-galactosidase activity assay the bacteria were grown in the ED minimal medium with either sulfate or methionine as sulfur source. **b.** exp = exponential growth phase, stat= stationary growth phase. **c.** BFS1851 = *mtnU::lacZ*, BSHP7020 = *mtnV::lacZ*, BSHP7014 = *mtnW::lacZ*, BFS1852 = *mtnX::lacZ*, BSHP7016 = *mtnY::lacZ*, BFS1853 = *mtnZ::lacZ*.

are nine putative paralogs in the genome of *B. subtilis* (YwfG, AlaT, AspB, PatA, YhdR, YdfD, PatB, YisV, and HisC). In the same way, MtnX (YkrX) is a member of the phosphatase family pfam00702 ([13], Fig. 6), and therefore of a ubiquitous class of hydrolases (several phosphatase genes in particular are present in the genome of *B. subtilis*). This is likely to account for the lack of phenotype under our growth conditions. Inactivation of *mtnZ* provides only a very weak, residual growth on MTR. Inactivation of *mtnK*, *mtnS*, *mtnY* and *mtnW* result in resistance to 3F-MTR and lack of growth on MTR. Inactivation of *mtnW* with a polar effect on the distal genes (by insertion of a disrupting plasmid) has a phenotype similar to that of *mtnY* (*i.e.* lack of growth on MTR, and lack of influence of MTR on sulfate supplemented plates). In contrast, we discovered that MTR is toxic when the distal genes are present (when used as sole sulfur source or even in the presence of sulfate, see Fig. 7). Because of the weak phenotype of a *mtnZ* mutant and the absence of phenotype of a *mtnX* mutant, we can be confident that MtnY acts before MtnW (this is a regular feature in operons, where it is often observed that the more distal genes code for proteins acting in the more proximate steps of the pathway).

The methionine salvage pathway has been deciphered in *K. pneumoniae*. It is possible, combining this knowledge to the genetic and physiologic results just described, to use it at the basis for reconstructing *in silico* the corresponding metabolic pathway in *B. subtilis*. The first steps are similar in both organisms: methylthioadenosine is converted into MTR by a nucleosidase (MtnA, [5]). Subsequently, MTR is phosphorylated into MTR-1-phosphate by MtnK [6]. On the other end of the pathway, methionine is synthesized directly from its keto acid precursor, 2-keto-4-methylthiobutyrate, by a transaminase. MtnV is the likely preferred enzyme for this activity. In *K. pneumoniae* a dioxygenase is converting 2,3-diketo-5-methylthio-1-phosphopentane into 2-keto-4-methylthiobutyrate [2]. Using dynamic programming (FASTA) we compared the sequence of the corresponding protein to the complete proteome of *B. subtilis*. YkrZ comes out as the first hit, as the most similar enzyme present in the proteome. Furthermore, it displays a strong consensus similarity with the dioxygenases of the family pfam03079 (Fig. 8) [13]. In order to check whether dioxygen was indeed involved in the case of *B. subtilis* we grew the cells anaerobically, with nitrate as an electron acceptor, and tested for growth on MTR: while the wild type strain grew well when sulfate was the carbon source, it failed to grow with MTR (Table 1).

Since this dioxygenase is coded in the *mtn* operon we can infer that it indeed displays the corresponding activity [12], and we therefore renamed it MtnZ. In *K. pneumoniae*, the immediate precursor activity is that of a coupled phosphatase. The presence of MtnX, which belongs to family pfam00702 comprising phosphatases is strongly suggestive of its involvement at this step [13]. We are thus left with two enzymes, and two steps. We also know, from the genetic data, that the steps are catalyzed in the order MtnY, MtnW. Finally, the reaction needed upstream of MtnZ is active on a molecule phosphorylated in position 1. MtnW is very similar to ribulosephosphate carboxylase oxygenase (Rubisco). It is therefore likely to be active on a ribulose-1-phosphate derivative. Hence MtnY, which is similar to the *araD* gene product of *E. coli* (ribulose-5-

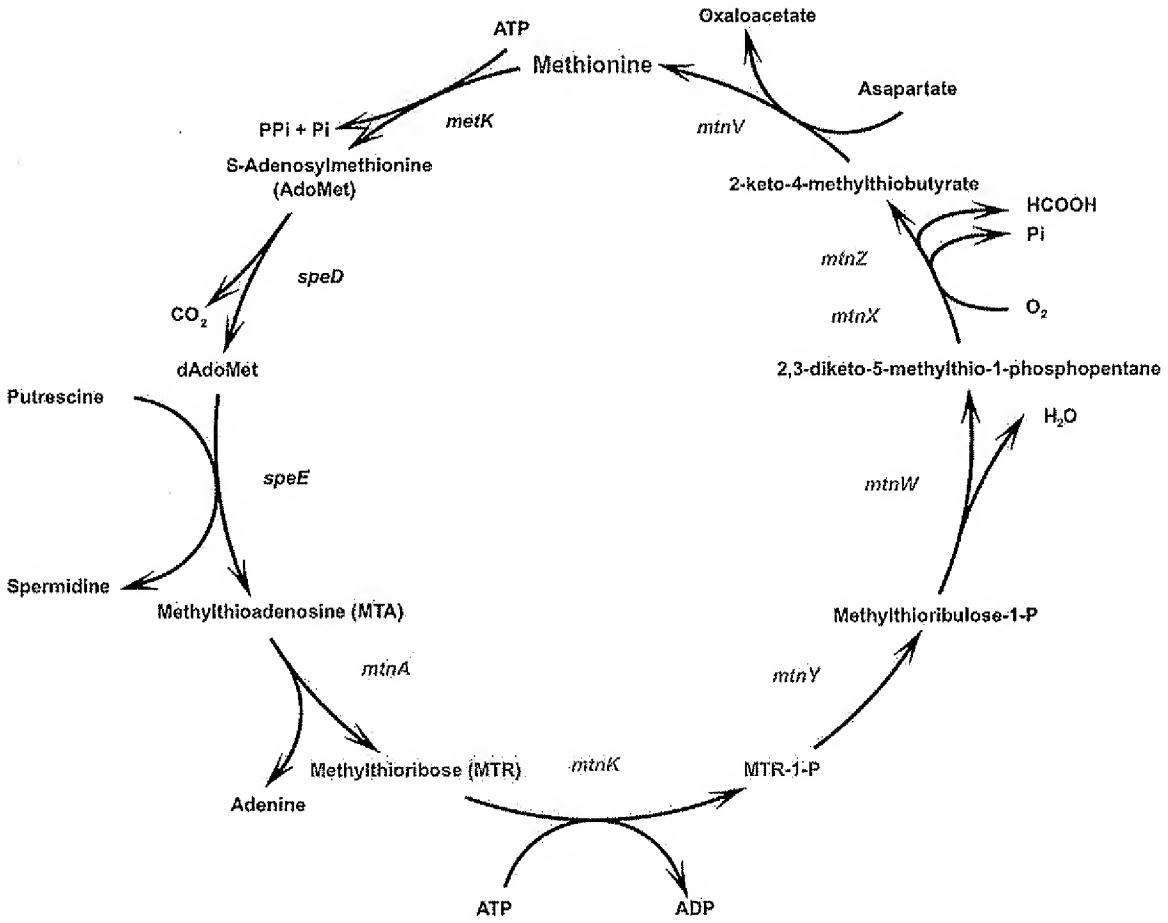


Figure 4
The MTR recycling pathway in *B. subtilis*.

phosphate epimerase) is most likely to be an epimerase that converts MTR-1-P into 5-thiomethyl-ribulose-1-phosphate, which is the substrate of MtnW. This is strongly supported by the list of similarities found about this gene at the SubtiList database [http://genolist.pasteur.fr/Subtilist] .

At this stage it is difficult to explicitly identify the activity of MtnW. Even in the case of the paradigmatic Rubisco, with many crystal structures known, the exact mechanism of catalysis is still a matter of controversy. However we can note (as did [8], at a time when they could not propose a function for the protein) that all the residues involved in catalysis have been conserved, the only residues modified being those involved in the binding of the phosphate at position 5 of ribulose diphosphate. The reaction is that of a dehydratase, but the pathway of the reaction is not yet known. Further work will establish the details of the reaction.

Finally MtnU is also defective for MTR recycling. However, this protein is synthesized at a level much lower that that of the other components of the pathway. We can therefore surmise that it is involved in a regulatory step in the pathway.

Discussion

Several genes in the vicinity of *mtnK* have been shown to have significant relationships with sulfur metabolism. In particular, it has been known for some time that genes *ykrWXYZ* were preceded by an S-box, typical of sulfur mediated regulation [7]. In addition, while analyzing the function of ribulose-1,5-diphosphate carboxylase (Rubisco), Hanson and Tabita discovered a class of highly related enzymes that were involved in sulfur metabolism [8].

Allowing cells to grow in the presence of the toxic MTR analog 3F-MTR, resistant mutants were found in genes *mtnK*, *mtnW* and *mtnY*. The trifluoromethyl group of 3F-MTR is the toxic group of the molecule and it was expected that

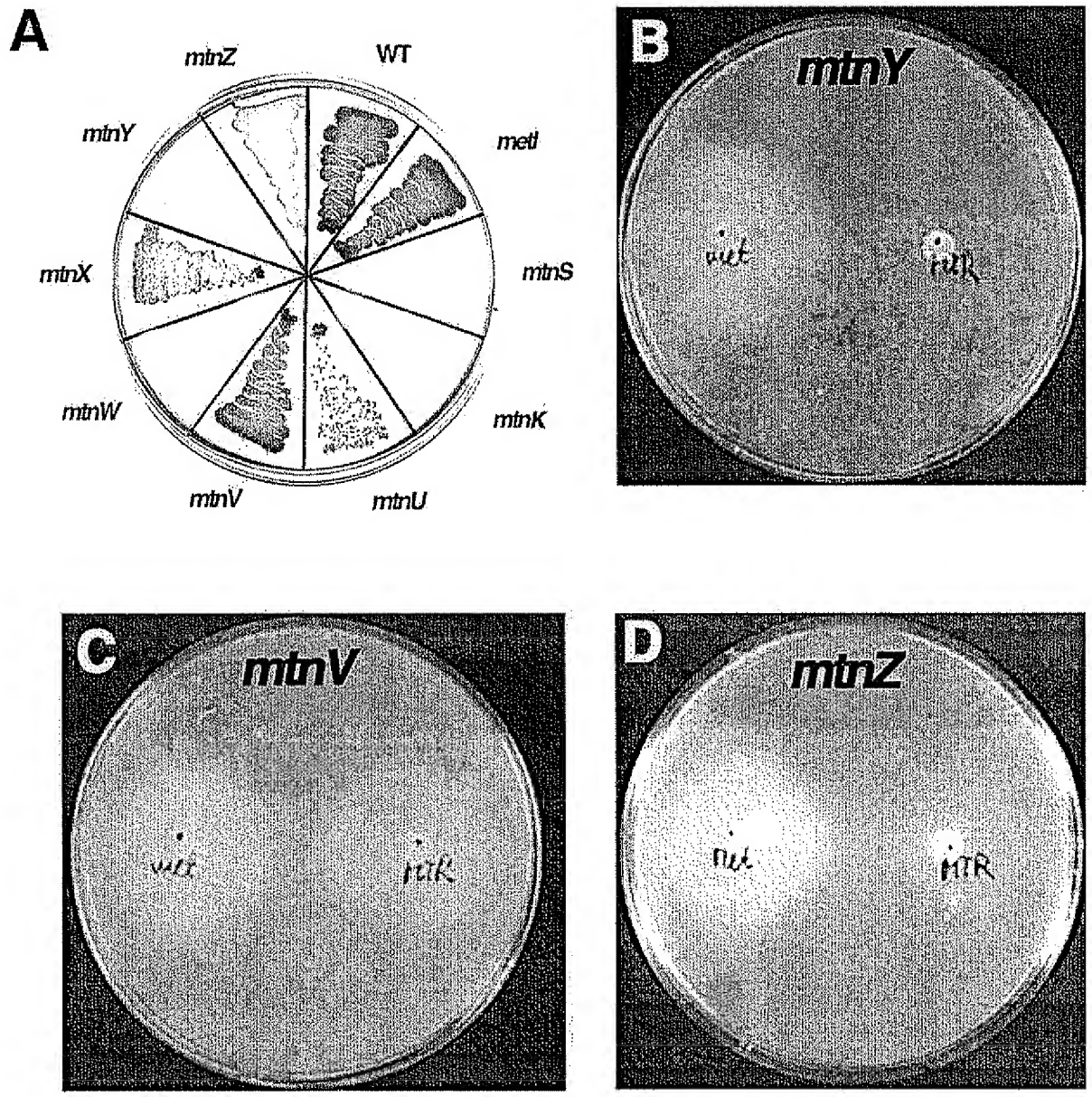


Figure 5
Growth of mutants from the *mtn* region with MTR as sole sulfur source. Panel A: ED1 minimal medium plate with 1 mM IPTG containing 0.2 mM MTR as sole sulfur source WT, *metI* (BSIP1143), *mtnS* (BSHP7010), *mtnK* (BFS1850), *mtnU* (BFS1851), *mtnV* (BSHP7020), *mtnW* (BSHP7014), *mtnX* (BFS1852), *mtnY* (BSHP7016) and *mtnZ* (BFS1853) were inoculated for over-night growth at 37°C. No growth of *mtnS*, *mtnK*, *mtnW* and *mtnY* is represented by an example of absence of growth around a disc with MTR of *mtnY* mutant in panelB. Normal growth of *mtnV* and *mtnX* is represented by an example of normal growth around a disc with MTR of *mtnV* mutant in panel C. The partial growth of the *mtnZ* mutant is illustrated by its growth around a disc with MTR in panel C. Panel B: The *mtnY* strain (BSHP7016) was inoculated on ED1 minimal medium plate with no added sulfur source. 10 µl of methionine (met) or MTR was put on paper discs and the plate was incubated over-night at 37°C. Panel C: The *mtnV* strain (BSHP7020) was inoculated on ED1 minimal medium plate with no added sulfur source. 10 µl of methionine (met) or MTR was put on paper discs and the plate was incubated over-night at 37°C. Panel D: The *mtnZ* strain (BFS1853) was inoculated on ED1 minimal medium plate with no added sulfur source. 10 µl of methionine (met) or MTR was adsorbed on paper discs and the plate was incubated over-night at 37°C. Methionine was used as a control.

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pfam00702
MtnX:   65   EEITSFVLEDAKIREGFREFVAFINEHEIPFYVISGGMDFFVYPLLEGIVEKDRIYCNHASF  122
Cons:   84   GEVLGLIALADKLYPGAREALKALKERGIKLAILTNGDRANAEAVLE-------LLGLADLF  134

MtnX:  123   DNDYIHIDWPHSCKGTCSNQCGCCKPSVIHELSEP----NQYIIMIGDSVTDVEAAKLSDL   183
Cons:  135   DVIVDSDDVGVG-KP---------KPEIFLLALERLGVKPEEVLMVGDGVNDAPALAAAGM   189
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Figure 6
Alignment of MtnX with the consensus of pfam00702 [http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=pfam00702&version=v1.54] , that includes L-2-haloacid dehalogenase, epoxide hydrolases and phosphatases. Red letters represent identities, blue letters conservative replacements (similarity classes: AGPST, ILMV, FWY, DENQ, HKR). A loop containing a metal (presumably iron, or an iron-sulfur cluster) is likely to be present in MtnX.

inactivation of any gene coding for the steps, including permeation, that led from MTR to methionine (the ultimate methyl donor, since AdoMet is an essential metabolite for the cell) would result in a resistant phenotype. Remarkably, no permease gene was found, suggesting that MTR enter the cells via several entries. In addition, apart from the *mtnKS* and *mtnWXYZ* operons no other genes was found, suggesting that all essential steps for recycling are coded for by these genes (or that other steps are coded for by redundant genes). The first step of the metabolic pathway is phosphorylation of MTR. The last step, presumably, is transamination, with *mtnV* being the preferred transaminase.

Interestingly, the pathway described in this work, although similar to that found by the pioneering work of Abeles and co-workers, uses an enzyme, MtnW, which is extremely similar to Rubisco but not present in *K. pneumoniae* or*K. oxytoca*[14,15]. While most of the genome sequence of this bacterium is known [http://wit.integratedgenomics.com/GOLD/] no counterpart of MtnW could be found (data not shown). The corresponding activity exists in *K. pneumoniae*, but no corresponding gene has yet been isolated. As discovered by Hanson and Tabita, MtnW counterparts constitute a special class (class IV) of Rubisco-like enzymes, which are involved in sulfur metabolism: we can presume that they are all part of the methionine salvage pathway in these organisms [8]. Interestingly, the expected reaction required to metabolize 5-thiomethyl-ribulose-1-phosphate is that of a dehydratase that may use a co-factor as a substrate for the reaction [16]. Rubisco, in the presence of carbon dioxide (resp. dioxygen), acts as a carboxylase (resp. dioxygenase) which cleaves the substrate. In the present case we expect that, instead of cleavage, we have maintenance of a five carbon molecule that is dephosphorylated (by MtnX) and subsequently cleaved by dioxygen in the reaction mediated by MtnZ. The counterpart of this activity in other genomes, including those with a methionine salvage pathway is not known.

As a strong support of this schema, we found counterparts of MtnK and of MtnZ in *K. pneumoniae*, substantiating the proposed pathway. In this latter organism the counterpart of MtnY is not known, and the corresponding step (opening of the MTR-1-P ring with epimerisation) is not known in any organism yet. MtnY is part of a very wide family of aldolases-epimerases-transketolases and *in silico* prediction of function alone, at this stage is highly problematic (wrong assignment is frequent for similar functions [17]), but combination with genetic data make the prediction highly probable [12]. We therefore propose that MtnY be used as a basis for annotation of similar gene products. For example in *Xylella fastidiosa*, gene XF2209 and in *Pseudomonas aeruginosa* gene PA1683 probably encodes the cognate activity. Noticeably, a counterpart exists in the Human Genome, where a similar pathway operates.

Two gene products are not directly accounted for in the present schema, MtnU and MtnS. MtnU is expressed at a very low level (ten times lower) as compared to MtnW, and this would hardly fit with the expected stoichiometric enzyme concentration usually found in multistep metabolic pathways. In addition, we found in this work that its synthesis is not submitted to any regulation by the sulfur source. Similarly, MtnS, which is highly similar to an eukaryotic translation initiation factor eIF-2B involved in GTP/GDP exchange is a member typical of a class of GTP-dependent regulators. The presence of two regulator molecules in this pathway indicates that it must have an important role in the cell. *B. subtilis* is likely to strive on the phylloplane. It is therefore regularly submitted to very high local concentrations of oxygen, and we speculate that this pathway, in addition to providing an excellent means to recycle the energy costly methionine, is used as a means to protect the cell against oxygen.

Table 3: Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Source or reference
Strains		
Escherichia coli		
TG1	K12 *supE hsdΔ5 thi Δ (lac-proAB)* F' [*traD36 proA+ proB+ lacI*^{q}*lacZΔM15*]	Laboratory collection
XL1-Blue	K12 *supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac-* F' [*proAB+ lacI*^{q}*lacZΔM15 Tn10*(tet^R)]	Laboratory collection
Bacillus subtilis		
168	*trpC2*	[31]
BSIP1143	*trpC2 metI::spc*	[32]
BSHP7010	*trpC2 mtnS::spc*	[6]
BFS1850	*trpC2mtnK::lacZ*	Functional analysis project[a][6]
BFS1851	*trpC2mtnU::lacZ*	Functional analysis project[a][6]
BFS1852	*trpC2mtnX::lacZ*	Functional analysis project[a]
BFS1853	*trpC2mtnZ::lacZ*	Functional analysis project[a]
BSHP7014	*trpC2mtnW::lacZ amyE::*(pxyl*mtnXYZ*)	This work
BSHP7015	*trpC2amyE::*(pxyl*mtnXYZ*)	This work
BSHP7016	*trpC2mtnY::lacZ*	This work
BSHP7020	*trpC2mtnV::lacZ*	This work
BSHP7064	*trpC2mtnW::Tn10*	This work
BSHP7065	*trpC2mtnY::Tn10*	This work
Plasmids		
pIC333	mini-*Tn10* delivery vector, Spc^R, Ery^R	[27]
pJM783	cloning vector, Cm^R, Amp^R	[25]
pX	cloning vector, Cm^R, Amp^R, pxyl promoter, *amyE* locus integration	[24]
pHPP7011	pJM *mtnV::lacZ*	This work
pHPP7014	pJM *mtnW::lacZ*	This work
pHPP7014bis	pJM *mtnW::lacZ* (*bla::spc*[b])	This work
pHPP7015	pX pxyl *mtnXYZ*	This work
pHPP7016	pJM *mtnY::lacZ*	This work

a. This strain has been constructed in the frame of the EC project for the functional characterization of the genome of *B. subtilis* in Europe. **b.***spc* is the spectinomycin resistance gene from *Staphylococcus aureus*.

Conclusions

This work demonstrates that a complete methionine salvage pathway exists in *B. subtilis*. This pathway is chemically similar to that in *K. pneumoniae*, but recruited different proteins to this purpose. In particular a paralogue or Rubisco, MtnW, is used at one of the steps in the pathway. A major observation stemming from the present experiments is that in the absence of MtnW MTR becomes extremely toxic to the cell. This sensitivity opens an unexpected target for new antimicrobial drugs, since analogs of 5-methylthio-ribulose1-phosphate might have a strong inhibitory effect on growth on bacteria containing this methionine salvage pathway, including *Bacillus anthracis*.

Materials and methods

Bacterial strains and plasmids, and growth media

E. coli and B. subtilis strains as well as plasmids used in this work are listed in Table 3. E. coli TG1 and XL1-Blue were used for cloning experiments (TG1 for single cross-over recombination and XL1-Blue for double cross-over recombination). Despite the fact that there are no public regulations yet in this domain in China, all experiments were performed in accordance with the European regulation requirements concerning the contained use of Genetically Modified Organisms of Group-I (French agreement N° 2735). E. coli and B. subtilis were grown in Luria-Bertani (LB) medium [18] and in ED minimal medium: K_2HPO_4, 8 mM; KH_2PO_4, 4,4 mM; glucose, 27 mM; Na_3-citrate, 0.3 mM; L-glutamine, 15 mM; L-tryptophan, 0.244 mM; ferric citrate, 33.5 μM; $MgSO_4$, 2 mM; $MgCl_2$, 0.61 mM; $CaCl_2$, 49.5 μM; $FeCl_3$, 49.9 μM; $MnCl_2$, 5.05 μM; $ZnCl_2$, 12.4 μM; $CuCl_2$, 2.52 μM; $CoCl_2$, 2.5 μM; Na_2MoO_4, 2.48 μM. When methionine was used as sulfur source (1 mM), $MgSO_4$ was replaced by $MgCl_2$ at the same magnesium concentration (2 mM). For assaying growth on plates, either the $MgSO_4$ containing medium or the sulfur-free basal medium was used ($MgSO_4$ was replaced by $MgCl_2$ as decribed above). In the latter case, 10 μl of the sulfur source under investigation was applicated onto paper discs (MTR, 200 mM stock solution and methionine, 100 mM stock solution) deposited at the center of the plate, after bacteria had been uniformly spread at the

surface of the plate, and growth was measured around the disk. In some cases MTR was used directly in the plate as sulfur source (0.2 mM). When necessary IPTG was included at 1 mM concentration. When xylose was added to the medium (0.5%) in order to trigger the expression of genes under the control of Pxyl inducible promoter, fructose was used as carbon source instead of glucose. LB and ED plates were prepared by addition of 17 g/liter Bacto agar or Agar Noble (Difco), respectively, to the medium. When included, antibiotics were added to the following concentrations: ampicillin, 100 mg/liter; chloramphenicol, 50 mg/liter; spectinomycin, 100 mg/liter; erythromycin plus lincomycin, 1 mg/liter and 25 mg/liter. Bacteria were grown at 37°C. The optical density (OD) of bacterial cultures was measured at 600 nm. MTR was prepared from MTA (Sigma, D5011) by acid hydrolysis as described by Schlenk [19]. 3-fluoromethythiorybose (3F-MTR, 5-thio-5-S-trifluoromethyl-D-ribose) was synthesised accordingly to [6,20]. For mutant selection, 3F-MTR was used at 100 mg/liter concentration in the ED minimal medium plates containing magnesium sulfate as sulfur source and glucose as carbon source. When applicated onto paper discs 10 µl of 100 mM stock solution of 3F-MTR was used. For anaerobic growth on plates, the Anaerocult A (Merck) within an anaerobiosis jar for CO2 production with simultanious O2 absorbtion was used. Sulfur-free ED minimal medium plates were supplemented to 1% glucose final concentrtion and with 0.5% sodium pyruvate and 20 mM sodium nitrate as electron acceptor. Plates were incubated at 37°C for 4 days with the sulfur source under investigation.

Transformation

Standard procedures were used to transform *E. coli*[21] and transformants were selected on LB plates containing ampicillin, spectinomycin or ampicillin plus spectinomycin. *B. subtilis* cells were transformed with plasmid DNA following the two-step protocol described previously [22]. Transformants were selected on LB plates containing erythromycin plus lincomycin or spectinomycin or chloramphenicol.

Molecular genetics procedures

Plasmid DNA was prepared from *E. coli* by standard procedures [21]. *B. subtilis* chromosomal DNA was purified as described by Saunders [23]. Restriction enzymes and T4 DNA ligase were used as specified by manufacturers.

DNA fragments used for cloning experiments were prepared by PCR using *Pfu*Turbo DNA polymerase (Stratagene). Amplified fragments were purified by QIAquick PCR Purification Kit (Qiagen). DNA fragments were purified from a gel using Spin-X columns from Corning Costar by subsequent centrifugation and precipitation.

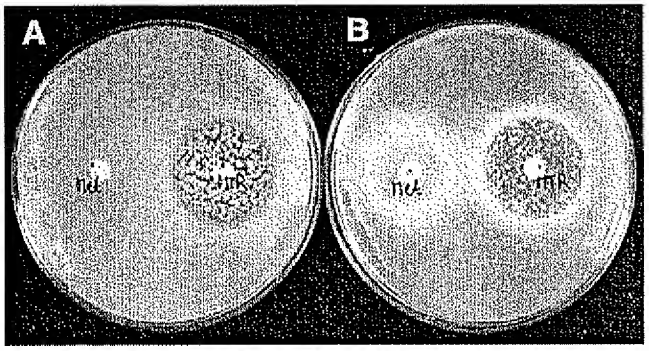

Figure 7
Toxicity of MTR for BSHP7014 strain. Strain BSHP7014 (*mtnW*::*lacZ amyE*::pxyl *mtnXYZ*) was grown on ED1 minimal medium plates in the presence of sulfate as sulfur source (panel A) or in the absence of any added sulfur source (agar as sole sulfur source, panel B). Xylose was added to the medium in order to trigger the expression of *mtnXYZ* from the pxyl promoter. 10 µl of methionine (met) or MTR was adsorbed on paper discs and plates were incubated overnight at 37°C. Methionine was used as a control for growth and/or toxicity of the sulfur source.

The *mtnXYZ* region (nucleotides -31 relative to the *mtnX* translation start point and ending 3 bp after the stop codon of *mtnZ*) was amplified by PCR using primers introducing a *Spe*I cloning site at the 5' end and a *Bam*HI cloning site at the 3' end of the fragment. This fragment was then inserted into the *Spe*I and *Bam*HI sites of xylose-inductible pX plasmid [24] producing plasmid pHPP7015. Prior to transformation, this plasmid was linearised at its unique *Sca*I site. Complete integration of the plasmid was obtained by a double cross-over event at the *amyE* locus, giving strain BSHP7015.

The DNA downstream from the *mtnW* gene (nucleotides +41 to +257 relative to the translation start point) was amplified by PCR using primers introducing an *Eco*RI cloning site at the 5' end and a *Bam*HI cloning site at the 3' end of the fragment, then inserted into the *Eco*RI and *Bam*HI sites of plasmid pJM783 [25] producing plasmid pHPP7014. To introduce an additional antibiotic resistance gene into plasmid pHPP7014, a *Sma*I restricted spectinomycin resistance cassette [26] was inserted into the *Sca*I restriction site of the *bla* gene producing plasmid pHPP7014bis. The plasmid in which the *mtnW* gene was disrupted as well as fused (transcriptional fusion) with the *lacZ* gene was introduced into the chromosome of BSHP7015 strain by a single cross-over event, giving strain BSHP7014.

For transcriptional fusion of *mtnY* with the *lacZ* gene, a DNA segment downstream from the *mtnY* gene (nucleotides + 57 to + 264 relative to the translation start point) was amplified by PCR using primers introducing an *Eco*RI

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pfam03079
MtnZ:   3    TIRIHDEANTTIENQEEVASFLDSQEVIYEQWDITRLPEHLSEKYDLTEEEKQQILDTFE   62
Cons:   1    QAWIMDDSEC--DQRLPHHTFPPEKAELDELAKLGVLYWKLDADDEETAEELLRIRKYRN   58

MtnZ:   63   TEIKDISTRRGYKAQDVISLSDSNPKLDELLENFKREHHHTDDEVRFIVSGHGIFVIQGQ   122
Cons:   59   YLDKDI---------DVTVCPETTPNFDEKLKKFFEEHLHTDEEIRYIVEGTGYFDVRDK   109

MtnZ:   123  DGTFFDVRLNPGDLISVPENIRHYFTLQEDRKVVAVRIFVTTEGWVP   169
Cons:   110  DDVWIRVLVEKGDLISLPAGIYHRFTTTPDNFVKALRLFVGKPGWTA   156
```

Figure 8
Alignment of MtnZ with the consensus of pfam03079 [http://www.ncbi.nlm.nih.gov/Structure/cdd/qrpsb.cgi?RID=1014604213-20481-4181] , coding for aci-reductone enzymes. Red letters represent identities, blue letters conservative replacements (classes: AGPST, ILMV, FWY, DENQ, HKR).

cloning site at the 5' end and a *Bam*HI cloning site at the 3' end of the fragment, then inserted into the *Eco*RI and *Bam*HI sites of plasmid pJM783 producing plasmid pHPP7016. The plasmid in which the *mtnY* gene was disrupted as well as fused (transcriptional fusion) with the *lacZ* gene was introduced into the chromosome by a single cross-over event, giving strain BSHP7016.

To construct a *mtnV* transcriptional fusion with the *lacZ* gene, a DNA fragment downstream from the *mtnV* gene (nucleotides + 44 to + 259 relative to the translation start point) was amplified by PCR using primers introducing an *Eco*RI cloning site at the 5' end and a *Bam*HI cloning site at the 3' end of the fragment, then inserted into the *Eco*RI and *Bam*HI sites of plasmid pJM783 producing plasmid pHPP7011. The plasmid in which the *mtnV* gene was disrupted as well as fused (transcriptional fusion) with the *lacZ* gene was introduced into the chromosome by a single cross-over event, giving strain BSHP7020.

Within the framework of a European Union and Japanese projects for the functional analysis of the genome of *B. subtilis*, more than 2000 genes have been disrupted by fusion with the *lacZ* reporter gene ([http://locus.jouy.inra.fr/cgi-bin/genmic/madbase/progs/madbase.operl] and [http://bacillus.genome.ad.jp]). The strains from the collection used in this study, constructed by Dr S. Krogh, are listed in Table 3.

Transposon mutagenesis

A transposon bank was constructed by introduction of the mini-Tn10 delivery vector pIC333 [27] into the *B. subtilis* 168 strain as described previously [28]. Several thousand independent clones were pooled together and 5 samples of chromosomal DNA were prepared for further use. To obtain 3F-MTR resistant clones, *B. subtilis* 168 was transformed with chromosomal DNA containing previously prepared transposon banks and clones were selected on LB plates containing spectinomycin. Then, using velvets replicas, clones were transferred onto minimal medium plates containing 3F-MTR at 100 µM concentration and allowed to grow for 24 hrs. The single transposon insertion event was confirmed by back-cross into strain 168 and check for 3F-MTR resistance. To determine the location of the transposon insertion, chromosomal DNA was prepared, followed by subsequent digestion with *Hin*dIII, self ligation in *E. coli* XL1-Blue strain and plasmid sequencing. The primers used for sequencing of transposon insertions were the followings: Tn10 left: 5'GGCCGATTCATTAATGCAGGG3' and Tn10 right: 5'CGATATTCACGGTTTACCCAC3'.

RNA isolation and manipulation

Total RNA was obtained from cells growing on ED1 minimal medium with sulfate or methionine as sulfur source to an OD_{600} of 0.5 using "High Pure RNA Isolation Kit" from Roche. The RNA concentration was determined by light absorption at 260 nm and 280 nm. 2 µg of RNA were loaded onto 1.2% agarose gel to check the RNA purity and integrity.

RNA molecules were separated on 1% agarose gels and transfered to nylon membranes (Hybond-N, Amersham). Efficiency of transfer was monitored by analysis of ethidium bromide-stained material. Membranes were prehybridized at 50°C for 1 hr in DIG Easy Hyb buffer from Roche. Hybridization was performed under the same conditions with *mtnV*, *mtnW* or *mtnZ* specific probes using a non-radioactive DNA labeling and detection kit "Dig-UTP labeling" from Roche.

Primer extension analysis using reverse transcriptase AMV (Roche) was performed as described by [29] with two oligonucleotides for each promoter identification. For *mtnKS* promoter the followings primers were used: 5'ACCAGCGTCTCGGCGCGAAAAAAATGCGCCCC3'

and 5'TCACAATGGAATTACGGTCGGTTGCTTTTGG3' (+137 to +169 and +172 to +203 with respect to the translation start point, respectively); for the *mtnU* promoter the followings primers were used: 5'AGTTCATCAAGATTGGCCAGATCATATCCG3' and 5'CAGGCAGAACAAGAACATCAGCATGTTTGC' (-133 to -103 and -90 to -60 with respect to translation start point, respectively); for the *mtnV* promoter the followings primers were used: 5'GTTTCATCTCCTCAACAATATGCTCAGGAG' and 5'TCCCAGATTGATAACGTCATGTCCTTCTGC' (-166 to -146 and -114 to -84 with respect to the translation start point, respectively); for the *mtnWXYZ* promoter the followings primers were used: 5'CGTTTCTCGTCCGAATCTTATCTCTCAGCC' and 5'AGCTGCAAGAATTAGCACCGTGCTTTATAAG' (+43 to +73 and +76 to +107 with respect to the translation start point, respectively). The same primers were used for the generation of sequence ladders. Reaction products were separated on 7% denaturing polyacrylamide gel containing 8 M urea. DNA sequences were determined using Sanger's dideoxy chain-termination method with "Thermo Sequenase radiolabeled terminator cycle sequencing kit" from Amersham Pharmacia Biotech.

Enzyme assays

B. subtilis cells containing *lacZ* fusions were assayed for β-galactosidase activity as described previously [30]. Specific activity was expressed in Units per mg protein. The Unit used is equivalent to 0.28 nmols min-1 at 28°C. Protein concentration was determined by Bradford's method using a protein assay Kit (Bio-Rad Laboratories). At least two independent cultures were monitored.

Amylase activity was detected after growth of *B. subtilis* strains on Tryptose Blood Agar Base (TBAB, Difco) supplemented with 10 g/liter hydrolyzed starch (Sigma). Starch degradation was detected by sublimating iodine onto the plates.

Abbreviations

bp: base pairs; CDS: coding sequence; IPTG: isopropyl β-D-thiogalactopyranoside; kb: kilobase; MTA: methylthioadenosine; MTR: methylthioribose; 3F-MTR: trifluoromethylthioribose; nt: nucleotides.

Authors' contributions

AS carried out the experimental part of the study, discovered the MTR toxic effect in the *mtnW* and *mtnU* mutants, drafted and wrote several sections of the manuscript.

AD outlined the rationale for the experiments, carried out the *in silico* part of the study and wrote the bulk of the manuscript.

Both authors read and approved the final manuscript.

Acknowledgements

We wish to thank Susan Krogh for the construction of the *mtnK*, *mtnU*, *mtnX* and *mtnZ* mutants during the *Bacillus subtilis* functional analysis project, and Sandrine Auger for providing the *metI* mutant.

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